BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/00, 9/88, A01H 5/00, 5/10

A1

(11) International Publication Number: WO 96/33270

(43) International Publication Date: 24 October 1996 (24.10.96)

(21) International Application Number: PCT/US96/05782

(22) International Filing Date: 19 April 1996 (19.04.96)

(30) Priority Data:

426,125	20 April 1995 (20.04.95)	US
455,355	31 May 1995 (31.05.95)	US

(60) Parent Applications or Grants
(63) Related by Continuation

US	08/426,125 (CIP)
Filed on	20 April 1995 (20.04.95)
US	08/455,355 (CIP)
Filed on	31 May 1995 (31.05.95)

(71) Applicant *(for all designated States except US)*: AMERICAN CYANAMID COMPANY [US/US]; One Cyanamid Plaza, Wayne, NJ 07470-8426 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: KAKEFUDA, Genichi [US/US]; 4 Westover Court, Yardley, PA 19067 (US). OTT, Karl-Heinz [DE/US]; 33 Cypress Court, Lawrenceville, NJ 08648 (US). KWAGH, Jae-Gyu [US/US]; 203 Yorkshire Lane, Fairless Hills, PA 19030 (US). STOCKTON, Gerald, W. [GB/US]; 391 South Milton Drive, Yardley, PA 19067 (US).

(74) Agents: ROBINSON, Joseph, R. et al.; Darby & Darby P.C., 805 Third Avenue, New York, NY 10022 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: STRUCTURE-BASED DESIGNED HERBICIDE RESISTANT PRODUCTS

(57) Abstract

Disclosed herein are structure-based modelling methods for the preparation of acetohydroxy acid synthase (AHAS) variants, including those that exhibit selectively increased resistance to herbicides such as imidazoline herbicides and AHAS inhibiting herbicides. The invention encompasses isolated DNAs encoding such variants, vectors that include the DNAs, and methods for producing the variant polypeptides and herbicide resistant plants containing specific AHAS gene mutations. Methods for weed control in crops are also provided.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

STRUCTURE-BASED DESIGNED HERBICIDE RESISTANT PRODUCTS

Field of the Invention

This invention pertains to structure-based modelling and design of variants of acetohydroxy acid synthase (AHAS) that are resistant to imidazolinones and other herbicides, the AHAS inhibiting herbicides, AHAS variants themselves, DNA encoding these variants, plants expressing these variants, and methods of weed management.

Background of the Invention

Acetohydroxy acid synthase (AHAS) is an enzyme that catalyzes the initial step in the biosynthesis of isoleucine, leucine, and valine in bacteria, yeast, and plants. For example, the mature AHAS from *Zea Mays* is approximately a 599-amino acid protein that is localized in the chloroplast (see Figure 1). The enzyme utilizes thiamine pyrophosphate (TPP) and flavin adenine dinucleotide (FAD) as cofactors and pyruvate as a substrate to form acetolactate. The enzyme also catalyzes the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate. AHAS is also known as acetolactate synthase or acetolactate pyruvate lyase (carboxylating), and is designated EC 4.1.3.18. The active enzyme is probably at least a homodimer. Ibdah et al. (*Protein Science*, 3:479-S, 1994), in an abstract, disclose one model for the active site of AHAS.

A variety of herbicides including imidazolinone compounds such as imazethapyr (PURSUIT® - American Cyanamid Company-Wayne, NJ), sulfonylurea-based compounds such as sulfometuron methyl (OUST® - E.I. du Pont de Nemours and

Company-Wilmington, DE), triazolopyrimidine sulfonamides (Broadstrike™ - Dow Elanco; see Gerwick, et al., *Pestic. Sci.* 29:357-364, 1990), sulfamoylureas (Rodaway et al., *Mechanisms of Selectively of Ac 322,140 in Paddy Rice, Wheat and Barley*, Proceedings of the Brighton Crop Protection Conference-Weeds, 1993), pyrimidyl-oxy-benzoic acids (STABLE© - Kumiai Chemical Industry Company, E.I. du Pont de Nemours and Company; see, The Pesticide Manual 10th Ed. pp. 888-889, Clive Tomlin, Ed., British Crop Protection Council, 49 Downing Street, Farmham, Surrey G49 7PH, UNITED KINGDOM), and sulfonylcarboxamides (Alvarado et al., U.S. Patent No. 4,883,914) act by inhibiting AHAS enzymatic activity. (See, Chaleff et al., *Science* 224:1443, 1984; LaRossa et al., *J.Biol.Chem.* 259:8753, 1984; Ray, *Plant Physiol.* 75:827, 11984; Shaner et al., *Plant Physiol.* 76:545, 1984). These herbicides are highly effective and environmentally benign. Their use in agriculture, however, is limited by their lack of selectivity, since crops as well as undesirable weeds are sensitive to the phytotoxic effects of these herbicides.

Bedbrook et al., U.S. Patent Nos. 5,013,659, 5,141,870, and 5,378,824, disclose several sulfonylurea resistant AHAS variants. However, these variants were either obtained by mutagenizing plants, seeds, or cells and selecting for herbicide-resistant mutants, or were derived from such mutants. This approach is unpredictable in that it relies (at least initially) on the random chance introduction of a relevant mutation, rather than a rational design approach based on a structural model of the target protein.

Thus, there is still a need in the art for methods and compositions that provide selective wide spectrum and/or specific herbicide resistance in cultivated crops. The present inventors have discovered that selective herbicide resistant variant forms of AHAS and plants containing the same can be prepared by structure-based modelling of AHAS against pyruvate oxidase (POX), identifying an herbicide binding pocket or pockets on the AHAS model, and designing specific mutations that alter the affinity of the herbicide for the binding pocket. These variants and plants are not inhibited or killed

by one or more classes of herbicides and retain sufficient AHAS enzymatic activity to support crop growth.

Brief Description of the Drawings

Figure 1 is an illustration of a 600 amino acid sequence corresponding to the approximately 599 amino acid sequence of acetohydroxy acid synthase (AHAS) from *Zea Mays* which is given as an example of a plant AHAS enzyme. The sequence does not include a transit sequence, and the extra glycine is vestigial from a thrombin cleavage site. Residues Met53, Arg128, and Phe135 are shown in bold.

Figure 2 is an illustration of the alignment of the sequence of maize AHAS and pyruvate oxidase (POX) from *Lactobacillus planarum*.

Figure 3 is a schematic representation of the secondary structure of an AHAS subunit. Regular secondary structure elements, α-helices and β-sheets, are depicted as circles and ellipses, respectively, and are numbered separately for each of the three domains within a subunit. Loops and coiled regions are represented by black lines, with numbers representing the approximate beginnings and ends of the elements. The locations of cofactor binding sites and known mutation sites are indicated by octahedrons and stars, respectively.

Figure 4 is an illustration of a computer-generated model of the active site of maize AHAS with imazethapyr (PURSUIT® herbicide) modeled into the binding pocket.

Figure 5 is an illustration of the homology among AHAS amino acid sequences derived from different plant species. pAC 751 is maize als 2 AHAS isozyme as expressed from the pAC 751 *E. Coli* expression vector as in Figure 1; Maize als 2 is the maize als 2 AHAS isozyme; Maize als 1 is the maize als 1 AHAS isozyme; Tobac 1 is the *tobacco* AHAS SuRA isozyme; Tobac 2 is the *tobacco* AHAS SuRB isozyme; Athcsr 12 is the *Arabidopsis thaliana* Csr 1.2 AHAS gene; Bnaal 3 is the *Brassica* napus AHAS III isozyme; and Bnaal 2 is the *Brassica* napus AHAS II isozyme.

pAC 751 and Maize als 2 are identical genes except that Maize als 2 starts at the beginning of the transit sequence and pAC 751 starts at the putative mature N-terminal site with an additional glycine at the N-terminal due to the thrombin recognition sequence in the pGEX-2T expression vector. The N-terminal glycine is not a natural amino acid at that position.

Amino acid sequence alignments of the AHAS proteins were generated by PILEUP (GCG Package - Genetics Computer Group, Inc., - University Research Park - Madison-WI). The consensus sequence was generated by PRETTY GCG Package.

Figure 6 is a photographic illustration of an SDS-polyacrylamide gel stained for protein showing purification of maize AHAS. The lanes contain (from left to right): A, Molecular weight markers; B, Crude *E. coli* cell extract; C, Glutathione-agarose affinity purified preparation; D, Thrombin digest of the affinity purified preparation; E, Second pass through glutathione-agarose column and Sephacryl S-100 gel filtration.

Figure 7 is a graphic illustration of the results of *in vitro* assays of the enzymatic activity of wild-type and mutant AHAS proteins in the absence and in the presence of increasing concentrations of imazethapyr (PURSUIT® herbicide). The Y axis represents the % of activity of the mutant enzyme, wherein the 100% value is measured in the absence of inhibitor.

Figure 8 is a graphic illustration of the results of *in vitro* assays of the enzymatic activity of wild-type and mutant AHAS proteins in the absence and presence of increasing concentrations of sulfometuron methyl (OUST® herbicide). The Y axis represents the % of activity of the mutant enzyme, wherein the 100% value is measured in the absence of inhibitor.

Figure 9 is a graphic illustration of *in vitro* assays of the enzymatic activity of wild-type *Arabidopsis* AHAS protein and the Met124Ile mutant *Arabidopsis* AHAS protein in the absence and presence of increasing concentrations of imazethapyr (PURSUIT® herbicide) and sulfometuron methyl (OUST® herbicide). The Y axis represents the % activity of the mutant

enzyme, wherein the 100% value is measured in the absence of inhibitor.

Figure 10 is a graphic illustration of *in vitro* assays of the enzymatic activity of wild-type *Arabidopsis* AHAS protein and the Met124His mutant *Arabidopsis* AHAS protein in the absence and presence of increasing concentrations of imazethapyr (PURSUIT® herbicide) and sulfometuron methyl (OUST® herbicide). The Y axis represents the % activity of the mutant enzyme, wherein the 100% value is measured in the absence of inhibitor.

Figure 11 is a graphic illustration of *in vitro* assays of the enzymatic activity of wild-type *Arabidopsis* AHAS protein and Arg199Glu mutant *Arabidopsis* AHAS protein in the absence and presence of increasing concentrations of imazethapyr (PURSUIT® herbicide) and sulfometuron methyl (OUST® herbicide). The Y axis represents the % activity of the mutant enzyme, wherein the 100% value is measured in the absence of inhibitor.

Figure 12 is a schematic illustration of a DNA vector used for plant transformation, which contains the nptII gene (encoding kanamycin resistance) under the control of the 35S promoter and an AHAS gene (wild type or variant) under the control of the *Arabidopsis* AHAS promoter.

Figure 13 is a photograph showing the root development of tobacco plants transformed with the *Arabidopsis* AHAS gene containing either the Met124Ile or Arg199Glu mutation and a non-transformed control. Plants were grown for 18 days after transfer into medium containing 0.25 μM imazethapyr.

Figure 14 is a photograph showing tobacco plants transformed with the *Arabidopsis* AHAS gene containing either the Met124Ile, Met 124His, or Arg199Glu mutation and a non-transformed control, which had been sprayed with twice the field rate (100 g/ha) of imazethapyr.

Figure 15 is a photograph showing the results of germination tests performed in the presence of the herbicide CL 299,263 (imazamox), which were performed on seeds harvested from primary tobacco plant transformants that had been

transformed with the *Arabidopsis* AHAS gene containing either the Met124Ile, Met 124His, or Arg199Glu mutation.

Summary of the Invention

The present invention provides a structure-based modelling method for the production of herbicide resistant AHAS variant protein. The method includes:

(a) aligning a target AHAS protein on pyruvate oxidase template or an AHAS modelling equivalent thereof to derive the three-dimensional structure of the target AHAS protein;

(b) modelling one or more herbicides into the three-dimensional structure to localize an herbicide binding pocket in the target AHAS protein;

(c) selecting as a target for a mutation, at least one amino acid position in the target AHAS protein, wherein the mutation alters the affinity of at least one herbicide for the binding pocket;

(d) mutating DNA encoding the target AHAS protein to produce a mutated DNA encoding a variant AHAS containing the mutation, such as, for example, at least one different amino acid, at the position; and

(e) expressing the mutated DNA in a first cell, under conditions in which the variant AHAS containing the mutation, such as, for example, the different amino acid(s), at the position is produced.

The method further may include:

(f) expressing DNA encoding wild-type AHAS protein parallel in a second cell;

(g) purifying the wild-type and the variant AHAS proteins from the cells;

(h) assaying the wild-type and the variant AHAS proteins for catalytic activity in conversion of pyruvate to acetolactate or in the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate, in the absence and in the presence of the herbicide; and

(i) repeating steps (c)-(h), wherein the DNA encoding the AHAS variant of step (e) is used as the AHAS-

encoding DNA in step (c) until a first herbicide resistant AHAS variant protein is identified having:

(i) in the absence of the at least one herbicide,

(a) catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

(b) catalytic activity in combination with any herbicide resistant AHAS variant protein also expressed in the cell, which may be the same as or different than the first AHAS variant protein, sufficient to maintain the viability of a cell in which it is expressed;

wherein the cell requires AHAS activity for viability; and

(ii) catalytic activity that is more resistant to the at least one herbicide than is wild-type AHAS.

An alternate structure-based modelling method for the production of herbicide resistant AHAS variant protein is also provided. This method includes:

(a) aligning a target AHAS protein on a first AHAS template derived from a polypeptide having the sequence of Figure 1 or a functional equivalent thereof to derive the three-dimensional structure of the target AHAS protein;

(b) modelling one or more herbicides into the three-dimensional structure to localize an herbicide binding pocket in the target AHAS protein;

(c) selecting as a target for a mutation, at least one amino acid position in the target AHAS protein, wherein the mutation alters the affinity of at least one herbicide for the binding pocket;

(d) mutating DNA encoding the target AHAS protein to produce a mutated DNA encoding a variant AHAS containing the mutation at the position; and

(e) expressing the mutated DNA in a first cell, under conditions in which the variant AHAS containing the mutation at the position is produced.

This method can further include:

(f) expressing DNA encoding wild-type AHAS protein in parallel in a second cell;

(g) purifying the wild-type and the variant AHAS protein from the cells;

(h) assaying the wild-type and the variant AHAS protein for catalytic activity in conversion of pyruvate to acetolactate or in the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate, in the absence and in the presence of the herbicide; and

(i) repeating steps (c)-(h), wherein the DNA encoding the AHAS variant of step (e) is used as the AHAS-encoding DNA in step (c) until a first herbicide resistant AHAS variant protein is identified having:

(i) in the absence of the at least one herbicide,

(a) catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

(b) catalytic activity in combination with any herbicide resistant AHAS variant protein also expressed in the cell, which may be the same as or different than the first AHAS variant protein, sufficient to maintain the viability of a cell in which it is expressed;

wherein the cell requires AHAS activity for viability; and

(ii) catalytic activity that is more resistant to the at least one herbicide than is wild-type AHAS.

In another alternate embodiment, the method includes:

(a) aligning a target AHAS protein on a first AHAS template having an identified herbicide binding pocket and having the sequence of Figure 1 or a functional equivalent thereof to derive the three-dimensional structure of the target AHAS protein;

(b) selecting as a target for a mutation, at least one amino acid position in the target AHAS protein, wherein the mutation alters the affinity of at least one herbicide for the binding pocket;

(c) mutating DNA encoding the target AHAS protein to produce a mutated DNA encoding a variant AHAS containing the mutation at the position; and

(d) expressing the mutated DNA in a first cell, under conditions in which the variant AHAS containing the mutation at the position is produced.

This method can further include:

(e) expressing DNA encoding wild-type target AHAS protein in parallel in a second cell;

(f) purifying the wild-type and the variant AHAS protein from the cells;

(g) assaying the wild-type and the variant AHAS protein for catalytic activity in conversion of pyruvate to acetolactate or in the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate, in the absence and in the presence of the herbicide; and

(h) repeating steps (b)-(g), wherein the DNA encoding the AHAS variant of step (d) is used as the AHAS-encoding DNA in step (b) until a first herbicide resistant AHAS variant protein is identified having:

(i) in the absence of the at least one herbicide,

(a) catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

(b) catalytic activity in combination with any herbicide resistant AHAS variant protein also expressed in the cell, which may be the same as or different than the first AHAS variant protein, sufficient to maintain the viability of a cell in which it is expressed;

wherein the cell requires AHAS activity for viability; and

(ii) catalytic activity that is more resistant to the at least one herbicide than is wild-type AHAS.

In preferred embodiments of the above methods, the catalytic activity in the absence of the herbicide is at least about 5% and most preferably is more than about 20% of the

catalytic activity of the wild-type AHAS. Where the herbicide is an imidazolinone herbicide, the herbicide resistant AHAS variant protein preferably has:

(i) catalytic activity in the absence of the herbicide of more than about 20% of the catalytic activity of the wild-type AHAS;

(ii) catalytic activity that is relatively more resistant to the presence of imidazolinone herbicides compared to wild-type AHAS; and

(iii) catalytic activity that is relatively more sensitive to the presence of sulfonylurea herbicides compared to imidazolinone herbicides.

The present invention further provides isolated DNA encoding acetohydroxy acid synthase (AHAS) variant proteins, the variant proteins comprising an AHAS protein modified by:

(i) substitution of at least one different amino acid residue at an amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing;

(ii) deletion of up to 5 amino acid residues preceding, or up to 5 amino acid residues following at least one amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261,

M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301,R302,F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing;

(iii) deletion of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(iv) addition of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(v) deletion of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1;

(vi) addition of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1; or

(vii) any combination of any of the foregoing.

In this numbering system, residue #2 corresponds to the putative amino terminus of the mature protein, i.e., after removal of a chloroplast targeting peptide.

The above modifications are directed to altering the ability of an herbicide, and preferably an imidazolinone-based herbicide, to inhibit the enzymatic activity of the protein. In a preferred embodiment, the isolated DNA encodes an herbicide-resistant variant of AHAS. Also provided are DNA vectors comprising DNA encoding these AHAS variants, variant AHAS proteins themselves, and cells, grown either *in vivo* or in cell culture, that express the AHAS variants or comprise these vectors.

In another aspect, the present invention provides a method for conferring herbicide resistance on a cell or cells and particularly a plant cell or cells such as, for example, a seed. An AHAS gene, preferably the *Arabidopsis thaliana* AHAS gene, is mutated to alter the ability of an herbicide to inhibit the enzymatic activity of the AHAS. The mutant gene is cloned into a compatible expression vector, and the gene is transformed into an herbicide-sensitive cell under conditions in which it is expressed at sufficient levels to confer herbicide resistance on the cell.

Also contemplated are methods for weed control, wherein a crop containing an herbicide resistant AHAS gene according to the present invention is cultivated and treated with a weed-controlling effective amount of the herbicide.

Also disclosed is a structure-based modelling method for the preparation of a first herbicide which inhibits AHAS activity. The method comprises:

(a) aligning a target AHAS protein on pyruvate oxidase template or an AHAS modelling functional equivalent thereof to derive the three-dimensional structure of the target AHAS protein;

(b) modelling a second herbicide having AHAS inhibiting activity into the three-dimensional structure to derive the location, structure, or a combination thereof of an herbicide binding pocket in the target AHAS protein; and

(c) designing a non-peptidic first herbicide which will interact with, and preferably will bind to, an AHAS activity inhibiting effective portion of the binding pocket, wherein the first herbicide inhibits the AHAS activity sufficiently to destroy the viability of a cell which requires AHAS activity for viability.

An alternative structure-based modelling method for the production of a first herbicide which inhibits AHAS activity, is also enclosed. The method comprises:

(a) aligning a target AHAS protein on a first AHAS template derived from a polypeptide having the sequence of Figure 1 or a functional equivalent thereof, to derive the three-dimensional structure of the target AHAS protein;

(b) modelling a second herbicide having AHAS inhibiting activity into the three-dimensional structure to derive the location, structure, or a combination thereof of an herbicide binding pocket in the target AHAS protein; and

(c) designing a non-peptidic first herbicide which will interact with, and preferably will bind to, an AHAS activity inhibiting effective portion of the binding pocket, wherein the first herbicide inhibits the AHAS activity sufficiently to destroy the viability of a cell which requires AHAS activity for viability.

Preferably in each method, the first herbicide contains at least one functional group that interacts with a functional group of the binding pocket.

Detailed Description of the Invention

The present invention encompasses the rational design or structure-based molecular modelling of modified versions of the enzyme AHAS and AHAS inhibiting herbicides. These modified enzymes (AHAS variant proteins) are resistant to the action of herbicides. The present invention also encompasses DNAs that encode these variants, vectors that include these DNAs, the AHAS variant proteins, and cells that express these variants. Additionally provided are methods for producing herbicide resistance in plants by expressing these variants and methods of weed control. The DNA and the AHAS variants of the present invention were discovered in studies that were based on molecular modelling of the structure of AHAS.

Rational Structure-Based Design of AHAS Variants and AHAS Inhibiting Herbicides

Herbicide-resistant variants of AHAS according to the present invention are useful in conferring herbicide resistance in plants and can be designed with the POX model, AHAS model, or functional equivalents thereof, such as, for example, transketolases, carboligases, pyruvate decarboxylase, proteins that bind FAD and/or TPP as a cofactor, or any proteins which have structural features similar to POX and/or AHAS; with an AHAS model such as a model having the sequence of Figure 1; or

with a functional equivalent of the sequence of Figure 1 including a variant modeled from a previous model. Proteins that can be used include any proteins having less than a root mean square deviation of less than 3.5 angstroms in their Cα carbons relative to any of the above-listed molecules. AHAS directed herbicides can be similarly modelled from these templates. A functional equivalent of an AHAS amino acid sequence is a sequence having substantial, i.e., 60-70%, homology, particularly in conserved regions such as, for example, a putative binding pocket. The degree of homology can be determined by simple alignment based on programs known in the art, such as, for example, GAP and PILEUP by GCG. Homology means identical amino acids or conservative substitutions. A functional equivalent of a particular amino acid residue in the AHAS protein of Figure 1 is an amino acid residue of another AHAS protein which when aligned with the sequence of Figure 1 by programs known in the art, such as, for example, GAP and PILEUP by GCG, is in the same position as the amino acid residue of Figure 1.

Rational design steps typically include: (1) alignment of a target AHAS protein with a POX backbone or structure or an AHAS backbone or structure; (2) optionally, and if the AHAS backbone has an identified herbicide binding pocket, modelling one or more herbicides into the three-dimensional structure to localize an herbicide binding pocket in the target protein; (3) selection of a mutation based upon the model; (4) site-directed mutagenesis; and (5) expression and purification of the variants. Additional steps can include (6) assaying of enzymatic properties and (7) evaluation of suitable variants by comparison to the properties of the wild-type AHAS. Each step is discussed separately below.

1. Molecular Modelling

Molecular modelling (and particularly protein homology modelling) techniques can provide an understanding of the structure and activity of a given protein. The structural model of a protein can be determined directly from experimental data such as x-ray crystallography, indirectly by homology

modelling or the like, or combinations thereof (See White, et al., *Annu. Rev. Biophys. Biomol. Struct.*, 23:349, 1994). Elucidation of the three-dimensional structure of AHAS provides a basis for the development of a rational scheme for mutation of particular amino acid residues within AHAS that confer herbicide resistance on the polypeptide.

Molecular modelling of the structure of *Zea mays* AHAS, using as a template the known X-ray crystal structure of related pyruvate oxidase (POX) from *Lactobacillus plantarum*, provides a three-dimensional model of AHAS structure that is useful for the design of herbicide-resistant AHAS variants or AHAS inhibiting herbicides. This modelling procedure takes advantage of the fact that AHAS and POX share a number of biochemical characteristics and may be derived from a common ancestral gene (Chang et al., *J.Bacteriol.* 170:3937, 1988).

Because of the high degree of cross-species homology in AHAS the modelled AHAS described herein or functional equivalents thereof can also be used as templates for AHAS variant protein design.

Derivation of one model using interactive molecular graphics and alignments is described in detail below. The three-dimensional AHAS structure that results from this procedure predicts the approximate spatial organization of the active site of the enzyme and of the binding site or pocket of inhibitors such as herbicides including, but not limited to, imidazolinone herbicides. The model is then refined and re-interpreted based on biochemical studies which are also described below.

Protein homology modelling requires the alignment of the primary sequence of the protein under study with a second protein whose crystal structure is known. Pyruvate oxidase (POX) was chosen for AHAS homology modelling because POX and AHAS share a number of biochemical characteristics. For example, both AHAS and POX share aspects of enzymatic reaction mechanisms, as well as cofactor and metal requirements. In both enzymes thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD), and a divalent cation are required for enzymatic activity. FAD mediates a redox reaction during

catalysis in POX but presumably has only a structural function in AHAS, which is possibly a vestigial remnant from the evolution of AHAS from POX. Both enzymes utilize pyruvate as a substrate and form hydroxyethyl thiamine pyrophosphate as a stable reaction intermediate (Schloss, J.V. et al. In *Biosynthesis of branched chain amino acids*, Barak, Z.J.M., Chipman, D.M., Schloss, J.V. (eds) VCH Publishers, Weinheim, Germany, 1990).

Additionally, AHAS activity is present in chimeric POX-AHAS proteins consisting of the N-terminal half of POX and the C-terminal half of AHAS, and there is a small degree of AHAS activity exhibited by POX itself. AHAS and POX also exhibit similar properties in solution (Risse, B. et al, *Protein Sci.* 1: 1699 and 1710, 1992; Singh, B.K., & Schmitt, G.K. (1989), *FEBS Letters*, 258: 113; Singh, B.K. et al. (1989) In: *Prospects for Amino Acid Biosynthesis Inhibitors in Crop Protection and Pharmaceutical Chemistry*, (Lopping, L.G., et al., eds., BCPC Monograph p. 87). With increasing protein concentration, both POX and AHAS undergo stepwise transitions from monomers to dimers and tetramers. Increases in FAD concentration also induce higher orders of subunit assembly. The tetrameric form of both proteins is most stable to heat and chemical denaturation.

Furthermore, the crystal structure of POX from *Lactobacillus planarum* had been solved by Muller et al., *Science* 259:965, 1993. The present inventors found that based in part upon the degree of physical, biochemical, and genetic homology between AHAS and POX, the X-ray crystal structure of POX could be used as a structural starting point for homology modelling of the AHAS structure.

AHAS and *L. plantarum* POX sequences were not similar enough for a completely computerized alignment, however. Overall, only about 20% of the amino acids are identical, while about 50% of the residues are of similar class (i.e. acidic, basic, aromatic, and the like). However, if the sequences are compared with respect to hydrophilic and hydrophobic residue classifications, over 500 of the 600 amino acids match. Secondary structure predictions for AHAS (Holley et al.,

Proc.Natl.Acad.Sci.USA 86:152, 1989) revealed a strong similarity to the actual secondary structure of POX. For nearly 70% of the residues, the predicted AHAS secondary structure matches that of POX.

POX monomers consist of three domains, all having a central, parallel β-sheet with crossovers consisting of α-helices and long loops. (Muller et al., *Science* 259:965, 1993). The topology of the sheets differs between the domains, i.e. in the first and third domains, the strands are assembled to the β-sheet in the sequence 2-1-3-4-6-5, while in the β-sheet of the second domain, the sequence reads 3-2-1-4-5-6.

Computer generated alignments were based on secondary structure prediction and sequence homology. The conventional pair-wise sequence alignment method described by Needleman and Wunch, *J. Mol. Biol*, 48: 443, 1970, was used. Two sequences were aligned to maximize the alignment score. The alignment score (homology score) is the sum of the scores for all pairs of aligned residues, plus an optional penalty for the introduction of gaps into the alignment. The score for the alignment of a pair of residues is a tabulated integer value. The homology scoring system is based on observing the frequency of divergence between a given pair of residues. (MO Dayhoff, RM Schwartz & BC Orcutt "Atlas of Protein Sequence and Structure" vol. 5 suppl. 3 pp. 345-362, 1978).

The alignments were further refined by repositioning gaps so as to conserve continuous regular secondary structures. Amino acid substitutions generated by evaluation of likely alignment schemes were compared by means of interactive molecular graphics. Alignments with the most conservative substitutions with respect to the particular functionality of the amino acids within a given site were chosen. The final alignment of both POX and AHAS is displayed in Figure 2. Conserved clusters of residues were identified, in particular for the TPP binding site and for parts of the FAD binding site. The alignment revealed a high similarity between AHAS and POX for the first domain, for most parts of the second domain, and for about half of the third domain. Most of the regions that aligned poorly and may fold differently in POX and in AHAS were

expected to be at the surface of the protein and were not involved in cofactor or inhibitor binding. The prediction of mutation sites is not substantially affected by small shifts in the alignment.

Most TPP binding residues are highly conserved between POX and AHAS (e.g. P48-G49-G50). In some cases, residues that were close to TPP differ between POX and AHAS but remain within a region that is highly conserved (for example, residues 90-110). On the other hand, the FAD binding site appeared to be less conserved. Although some FAD binding residues were strongly conserved (for example, D325-I326-D327-P328), others clearly differed between AHAS and POX (for example, residues in the loop from positions 278 to 285 are not homologous. A detailed analysis revealed that, at least for some of the less-conserved contact sites, the interactions were mediated by the polypeptide backbone rather than by the side chains. Hence, conservation was only required for the polypeptide fold and was not required for the amino acid sequence (for example, the backbone of residues 258-263 binds the ribitol chain of FAD). One half of the adenine and the isoalloxazine binding sites clearly differ.

After aligning the primary structure, a homology model was built by transposition of AHAS amino acid sequences to the POX template structure. Missing coordinates were built stepwise using templates of amino acid residues to complete undefined side chains. Data bank searches and energy minimization of small parts of the molecule were used to complete the conformations of undefined loop regions. The cofactors TPP and FAD were modeled into their binding pockets. This model was then subjected to a complete, 5000 cycle energy minimization. All computer modelling was performed in an IRIS Indigo Elan R4000 Workstation from Silicon Graphics Co. Interactive molecular modelling and energy-minimization were performed using Quanta/CHARMm 4.0 from Molecular Simulations Inc. During this step, the conformation was stable, indicating that no strongly disfavored interactions, such as, for example, close van der Waals contacts, had occurred. The results are shown schematically in Figure 3.

Characteristics of Predicted AHAS Structure

Inspection of the modelled AHAS structure described above revealed that most of the protein folds with a backbone that is energetically reasonable, with most hydrophilic side chains accessible to the solvent. The surface of the β-sheets are smooth and accommodate the cross-over regions that are attached to them.

A model for dimeric AHAS was generated by duplicating the coordinates of the energy minimized monomeric AHAS and superimposing the two copies on two POX subunits using pairs of Cα coordinates as defined in the alignment scheme. The polypeptide chain of AHAS folds into three similarly folded domains composed of a six-stranded parallel β-sheet core surrounded by long "loops" and α-helices. Two subunits are assembled such that the first domain of one subunit is in close proximity to the cofactor-binding domains 2 and 3 of the other subunit. A solvent-filled space remains between the subunits at this site. This pocket, which is defined by the confluence of the three domains, is the proposed entry site for the substrate. It is also proposed to be the binding site for herbicides.

The inner surface of the binding pocket is outlined by the cofactors. The thiazol of TPP is positioned at the bottom of the pocket. Domain 3 contributes to the inner surface of the pocket with a short α-helix that points its axis towards the pyrophosphate of TPP, compensating the phosphate charges with its dipolar moment. This critical helix, which starts with G498, a "turn" residue in close contact with TPP, and which ends at F507, contains three known mutation sites for sulfonylurea resistance: V500, W503, and F507 (See, U.S. Patent Nos. 5,013,659; 5,141,870; and 5,378,824). In domain 1, the loop defined as P48-S52 (between β-strand 2 and α-helix 2) faces W503, a mutation in which confers resistance to imidazolinones. Residues Y47 to G50 are also in contact with TPP. This loop is adjacent to P184-Q189, another turn, which connects the last strand of the β-sheet of domain 1 with a β-strand that connects with domain 2. Within the pocket, near its entrance, is a long region of domain 1 that interacts with a

complementary stretch of domain 2. Residues 125-129 and 133-137 of domain 1 and residues 304-313 of domain 2 are at the surface of the pocket. A turn consisting of T96-G100 is between loop 125-129 and TPP. A further stretch of domain 3 and two regions of domain 2 that line the binding pocket are at the opposite corner of the pocket. Residues 572, 575, 582, and 583 of domain 3 define the pocket surface on one side. The remaining part of the interior of the pocket's surface is defined by FAD and by a loop, L278-G282, that contacts the isoalloxazine ring of FAD.

The structural models of the AHAS protein can also be used for the rational design of herbicides or AHAS inhibitors.

2. Modelling of Herbicides Into Binding Sites

Imazethapyr, the active imidazolinone in PURSUIT®, was positioned into its proposed binding site using interactive molecular graphics (Figure 4) and the software described above (Figure 4). K185 was chosen as an "anchor" to interact with the charge of the carboxyl group. The imidazolinone's NH-CO unit was placed to form hydrogen bonds to G50 and A51. This positioned the methyl substitute of imazethapyr close to V500 on the backbone of the small α-helix. The isopropyl group is possibly bound by hydrophobic residues of the amino acids in the region of residues 125-135 that contribute to the inner surface of the pocket. The pyridine ring is most probably "sandwiched" between A134 or F135, F507 and W503. W503 also interacts with the imidazolinone ring system.

In a similar fashion, the sulfonylurea herbicides were modelled into a site that partially overlapped the described imidazolinone binding site. Overlap of sulfonylurea and imidazolinone binding sites was consistent with competition binding experiments and with established mutant data, which show that the same mutation in maize, W503L, can confer resistance to both herbicides. In these models, most of the known mutation sites that confer sulfonylurea herbicide resistance, i.e. G50, A51, K185, V500, W503, F507, are in close contact to the bound herbicides. P126 and A51 are required for keeping the K185 side chain in place by generating a

hydrophobic pore. S582, a site for specific imidazolinone resistance, is distant from the binding region and is located in the region where the homology is so poor that a change in the fold is expected. The FAD binding site apparently has low homology between AHAS and POX in this region; S582 is a residue that confers resistance in maize, and that S582 and its adjacent residues are in close contact to the active site pocket. It is proposed that FAD and the loop region encompassing residues 278 to 285 move slightly away from the third domain, (downward in Figure 4) and that a loop that contains S582 folds into the space between the helix at positions 499 to 507 and the loop at positions 278 to 285. D305, another known resistance site, is close to FAD and modulates the interaction between domains 1 and 2. M280 may either be involved in positioning of the helix at positions 498 to 507 or directly in inhibitor binding. M280 and D305 could also be directly involved in inhibitor binding if domains 1 and 2 move slightly closer to each other.

3. Selection of Mutations

Specific amino acid residues are pinpointed as sites for the introduction of mutations into the primary sequence of AHAS. These amino acids are selected based upon their position in that if that amino acid residue position is modified, there will be a resultant alteration (i.e. decline) in the affinity of an herbicide for the binding pocket. It is not necessary that the mutation position reside in the binding pocket as amino acid residues outside the pocket itself can alter the pocket charge or configuration. The selection of target sites for mutation is achieved using molecular models as described above. For example according to the model above, arginine at position 128 (designated R128 in Figure 1 using the single-letter code for amino acids) is located near the entrance to the substrate- and herbicide-binding pocket and has a large degree of conformational freedom that may allow it to participate in transport of charged herbicides into the binding pocket. Therefore, this residue is substituted by alanine to remove both its charge and its long hydrophobic side chain. (The resulting mutation is designated R128A).

The mutations may comprise simple substitutions, which replace the wild-type sequence with any other amino acid. Alternatively, the mutations may comprise deletions or additions of one or more amino acids, preferably up to 5, at a given site. The added sequence may comprise an amino acid sequence known to exist in another protein, or may comprise a completely synthetic sequence. Furthermore, more than one mutation and/or more than one type of mutation may be introduced into a single polypeptide.

4. Site-Directed Mutagenesis

The DNA encoding AHAS can be manipulated so as to introduce the desired mutations. Mutagenesis is carried out using methods that are standard in the art, as described in, for example, Higuchi, R., Recombinant PCR, In M.A. Innis, et al., eds, *PCR Protocols: A Guide to Methods and Applications*, Academic Press, pp. 177-183, 1990.

5. Expression and Purification of Variants

The mutated or variant AHAS sequence is cloned into a DNA expression vector (see, e.g., Example 3) and is expressed in a suitable cell such as, for example, *E. coli*. Preferably, the DNA encoding AHAS is linked to a transcription regulatory element, and the variant AHAS is expressed as part of a fusion protein, for example, glutathione-S-transferase, to facilitate purification (see Example 3 below). The variant AHAS is then purified using affinity chromatography or any other suitable method known in the art. "Purification" of an AHAS polypeptide refers to the isolation of the AHAS polypeptide in a form that allows its enzymatic activity to be measured without interference by other components of the cell in which the polypeptide is expressed.

6. Assaying of Enzymatic Properties

The purified variant AHAS may be assayed for one or more of the following three properties:

(a) specific or catalytic activity for conversion of pyruvate to acetolactate (expressed as units/mg pure AHAS, wherein a unit of activity is defined as 1 μmole acetolactate produced/hour), or for condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate (expressed as

units/mg pure AHAS, wherein a unit of activity is defined as 1 μ mole acetohydroxybutyrate produced/hr.;

(b) level of inhibition by herbicide, such as, for example, imidazolinone (expressed as IC_{50}, the concentration at which 50% of the activity of the enzyme is inhibited); and

(c) selectivity of resistance to the selected herbicide vs. other herbicides. The selectivity index is defined as the fold resistance of the mutant to imidazolinones relative to the wild-type enzyme, divided by the fold resistance of the same mutant to other herbicides also relative to the wild-type). Fold resistance to an herbicide relative to the wild-type enzyme is expressed as the IC_{50} of variant, divided by the IC_{50} of the wild type. The selectivity index (S.I.) is thus represented by the following equation:

$$S.I. \frac{IC_{50} \text{ of variant for herb.A}}{IC_{50} \text{ of wild type for herb.A}} / \frac{IC_{50} \text{ of variant for herb.B}}{IC_{50} \text{ of wild type for herb.B}}$$

Suitable assay systems for making these determinations include, but are not limited to, those described in detail in Example 4 below.

7.a. Evaluation of Suitable Variants

The enzymatic properties of variant AHAS polypeptides are compared to the wild-type AHAS. Preferably, a given mutation results in an AHAS variant polypeptide that retains *in vitro* enzymatic activity towards pyruvate or pyruvate and 2-ketobutyrate, i.e., the conversion of pyruvate to acetolactate or in the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate (and thus is expected to be biologically active *in vivo*), while exhibiting catalytic activity that is relatively more resistant to the selected herbicide(s) than is wild-type AHAS. Preferably, the variant AHAS exhibits:

(i) in the absence of the at least one herbicide,

(a) catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

(b) catalytic activity in combination with any herbicide resistant AHAS variant protein also

expressed in the cell, which may be the same as or different than the first AHAS variant protein, sufficient to maintain the viability of a cell in which it is expressed;

wherein the cell requires AHAS activity for viability; and

(ii) catalytic activity that is more resistant to the at least one herbicide than is wild type AHAS;

and that is relatively more resistant to the herbicide(s) than is wild-type AHAS.

Therefore, any one specific AHAS variant protein need not have the total catalytic activity necessary to maintain the viability of the cell, but must have some catalytic activity in an amount, alone or in combination with the catalytic activity of additional copies of the same AHAS variant and/or the catalytic activity of other AHAS variant protein(s), sufficient to maintain the viability of a cell that requires AHAS activity for viability. For example, catalytic activity may be increased to minimum acceptable levels by introducing multiple copies of a variant encoding gene into the cell or by introducing the gene which further includes a relatively strong promoter to enhance the production of the variant.

More resistant means that the catalytic activity of the variant is diminished by the herbicide(s), if at all, to a lesser degree than wild-type AHAS catalytic activity is diminished by the herbicide(s). Preferred more resistant variant AHAS retains sufficient catalytic to maintain the viability of a cell, plant, or organism wherein at the same concentration of the same herbicide(s), wild-type AHAS would not retain sufficient catalytic activity to maintain the viability of the cell, plant, or organism.

Preferably the catalytic activity in the absence of herbicide(s) is at least about 5% and, most preferably, is more than about 20% of the catalytic activity of the wild-type AHAS in the absence of herbicide(s). Most preferred AHAS variants are more resistant to imidazolinone herbicides than to other herbicides such as sulfonylurea-based herbicides, though in some applications selectivity is neither needed nor preferred.

In the case of imidazolinone-resistant variant AHAS, it is preferred that the AHAS variant protein has

(i) catalytic activity in the absence of said herbicide of more than about 20% of the catalytic activity of said wild-type AHAS;

(ii) catalytic activity that is relatively more resistant to presence of imidazolinone herbicides compared to wild type AHAS; and

(iii) catalytic activity that is relatively more sensitive to the presence of sulfonylurea herbicides compared to imidazolinone herbicides. Most preferred herbicide-resistant AHAS variants exhibit a minimum specific activity of about 20 units/mg, minimal or no inhibition by imidazolinone, and a selectivity index ranging from about 1.3 to about 3000 relative to other herbicides.

Without wishing to be bound by theory, it is believed that systematic and iterative application of this method to wild type or other target AHAS protein will result in the production of AHAS variants having the desired properties of high enzymatic activity as explained above and resistance to one or more classes of herbicides. For example, mutation of a wild-type AHAS sequence at a particular position to a given amino acid may result in a mutant that exhibits a high degree of herbicide resistance but a significant loss of enzymatic activity towards pyruvate or pyruvate and 2-ketobutyrate. In a second application of the above method, the starting or target AHAS polypeptide would then be this variant (in place of the wild-type AHAS). Rational design then involves substituting other amino acids at the originally mutated position and/or adding or deleting amino acids at selected points or ranges in the expectation of retaining herbicide resistance but also maintaining a higher level of enzymatic activity.

The structure-based rational design of herbicide resistant AHAS proteins offers many advantages over conventional approaches that rely on random mutagenesis and selection. For example, when substitution of a particular amino acid with another requires substitution of more than one

nucleotide within the codon, the likelihood of this occurring randomly is so low as to be impractical. By contrast, even double or triple changes in nucleotide sequence within a codon can be easily implemented when suggested by a rational design approach. For example, one rationally designed mutation to confer selective imidazolinone resistance requires a change from arginine to glutamate. Arginine is encoded by CGT, CGC, CGA, CGG, AGA, AGG, while glutamate is encoded by GAA and GAG. Since none of the arginine codons begins with GA, this mutation would require a double substitution of adjacent nucleotides which would occur so rarely using random mutagenesis as to be unpredictable and unrepeatable with any certainty of success. Although mutation frequency can be increased during random mutagenesis, alterations in nucleotide sequence would have an equal probability of occurring throughout the AHAS gene, in the absence of prior site-direction of the mutations. This increases the chance of obtaining an irrelevant mutation that interferes with enzymatic activity. Similarly, it would be rare, using random mutagenesis, to find a multiple amino acid substitution, deletion, or substitution/deletion mutation that confers herbicide resistance while maintaining catalytic activity. Deletion mutations that confer herbicide resistance would also be unlikely using a random mutagenesis approach. Deletions would need to be limited to small regions and would have to occur in triplets so as to retain the AHAS reading frame in order to retain enzymatic activity.

However, with a rational structure-based approach, double amino acid substitution and/or deletion mutations are relatively easily achieved and precisely targeted. Furthermore, different mutagens used in random mutagenesis create specific types of mutations. For example, sodium azide creates point substitution mutations in plants, while radiation tends to create deletions. Accordingly, two mutagenesis protocols would have to be employed to obtain a multiple combination substitution/deletion.

Finally, the present structure-based method for rational design of herbicide-resistant AHAS variants allows for iterative improvement of herbicide resistance mutations, a step

that is not facilitated by random mutagenesis. Identification of a mutation site for herbicide resistance by random mutagenesis may offer little, if any, predictive value for guiding further improvements in the characteristics of the mutant. The present structure-based approach, on the other hand, allows improvements to be implemented based on the position, environment, and function of the amino acid position in the structural model.

The iterative improvement method also allows the independent manipulation of three important properties of AHAS: level of resistance, selectivity of resistance, and catalytic efficiency. For example, compensatory mutations can be designed in a predictive manner. If a particular mutation has a deleterious effect on the activity of an enzyme, a second compensatory mutation may be used to restore activity. For example, a change in the net charge within a domain when a charged residue is introduced or lost due to a mutation can be compensated by introducing a second mutation. Prediction of the position and type of residue(s) to introduce, delete, or substitute at the second site in order to restore enzymatic activity requires a knowledge of structure-function relationships derived from a model such as that described herein.

7.b. Design of Non-Peptide Herbicides or AHAS Inhibitors

A chemical entity that alters and may fit into the active site of the target protein or bind in any position where it could inhibit activity may be designed by methods known in the art, such as, for example, computer design programs that assist in the design of compounds that specifically interact with a receptor site.

An example of such a program is LUDI (Biosym Technologies - San Diego, CA) (see also, Lam, et al., *Science* 263:380, 1994; Thompson, et al., *J. Med. Chem.*, 37:3100, 1994).

The binding pocket and particularly the amino acid residues that have been identified as being involved as inhibitor binding can be used as anchor points for inhibitor design.

The design of site-specific herbicides is advantageous in the control of weed species that may spontaneously develop herbicide resistance in the field, particularly due to mutations in the AHAS gene.

Herbicide-Resistant AHAS Variants: DNA, Vectors, and Polypeptides

The present invention also encompasses isolated DNA molecules encoding variant herbicide-resistant AHAS polypeptides. Genes encoding AHAS polypeptides according to the present invention may be derived from any species and preferably a plant species, and mutations conferring herbicide resistance may be introduced at equivalent positions within any of these AHAS genes. The equivalence of a given codon position in different AHAS genes is a function of both the conservation of primary amino acid sequence and its protein and the retention of similar three-dimensional structure. For example, Figure 5 illustrates the high degree of sequence homology between AHAS polypeptides derived from different plant species. These AHAS polypeptides exhibit at least about 60 to about 70% overall homology. Without wishing to be bound by theory, it is believed that in regions of the polypeptide having a highly conserved sequence, the polypeptide chain conformation will also be preserved. Thus, it is possible to use an AHAS-encoding sequence from one species for molecular modelling, to introduce mutations predictively into an AHAS gene from a second species for initial testing and iterative improvement, and finally, to introduce the optimized mutations into AHAS derived from yet a third plant species for expression in a transgenic plant.

In one series of embodiment, these AHAS DNAs encode variants of an AHAS polypeptide and preferably of the maize AHAS polypeptide of Figure 1 in which the polypeptide is modified by substitution at or deletion preceding or following one or more of Figure 1 amino acid residues P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281,

G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing; insertions or deletions between Figure 1 Q124 and H150 or functional equivalents thereof; insertions or deletions between Figure 1 G300 and D324 or functional equivalents thereof; and any combination of any of the foregoing thereof.

The mutations, whether introduced into the polypeptide of Figure 1 or at equivalent positions in another plant AHAS gene, may comprise alterations in DNA sequence that result in a simple substitution of any one or more other amino acids or deletions of up to 5 amino acid residues proceeding or up to 5 amino acids residues following any of the residence listed above. Suitable amino acid substituents include, but are not limited to, naturally occurring amino acids.

Alternatively, the mutations may comprise alterations in DNA sequence such that one or more amino acids are added or deleted in frame at the above positions. Preferably, additions comprise about 3 to about 30 nucleotides, and deletions comprise about 3 to about 30 nucleotides. Furthermore, a single mutant polypeptide may contain more than one similar or different mutation.

The present invention encompasses DNA and corresponding RNA sequences, as well as sense and antisense sequences. Nucleic acid sequences encoding AHAS polypeptides may be flanked by natural AHAS regulatory sequences, or may be associated with heterologous sequences, including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns, 5'- and 3'- noncoding regions, and the like. Furthermore, the nucleic acids can be modified to alter stability, solubility, binding affinity and specificity. For

example, variant AHAS-encoding sequences can be selectively methylated. The nucleic acid sequences of the present invention may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

The invention also provides vectors comprising nucleic acids encoding AHAS variants. A large number of vectors, including plasmid and fungal vectors, have been described for expression in a variety of eukaryotic and prokaryotic hosts. Advantageously, vectors may also include a promotor operably linked to the AHAS encoding portion. The encoded AHAS may be expressed by using any suitable vectors and host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Examples of suitable vectors include without limitation pBIN-based vectors, pBluescript vectors, and pGEM vectors.

The present invention also encompasses both variant herbicide-resistant AHAS polypeptides or peptide fragments thereof. As explained above, the variant AHAS polypeptides may be derived from the maize polypeptide shown in Figure 1 or from any plant or microbial AHAS polypeptide, preferably plant AHAS polypeptide. The polypeptides may be further modified by, for example, phosphorylation, sulfation, acylation, glycosylation, or other protein modifications. The polypeptides may be isolated from plants, or from heterologous organisms or cells (including, but not limited to, bacteria, yeast, insect, plant, and mammalian cells) into which the gene encoding a variant AHAS polypeptide has been introduced and expressed. Furthermore, AHAS polypeptides may be modified with a label capable of providing a detectable signal, either directly or indirectly, including radioisotopes, fluorescent compounds, and the like.

Chemical-resistant Plants and Plants Containing Variant AHAS Genes

The present invention encompasses transgenic cells, including, but not limited to seeds, organisms, and plants into

which genes encoding herbicide-resistant AHAS variants have been introduced. Non-limiting examples of suitable recipient plants are listed in Table 1 below:

TABLE 1

RECIPIENT PLANTS

COMMON NAME	FAMILY	LATIN NAME
Maize	Gramineae	Zea mays
Maize, Dent	Gramineae	Zea mays dentiformis
Maize, Flint	Gramineae	Zea mays vulgaris
Maize, Pop	Gramineae	Zea mays microsperma
Maize, Soft	Gramineae	Zea mays amylacea
Maize, Sweet	Gramineae	Zea mays amyleasaccharata
Maize, Sweet	Gramineae	Zea mays saccharate
Maize, Waxy	Gramineae	Zea mays ceratina
Wheat, Dinkel	Pooideae	Triticum spelta
Wheat, Durum	Pooideae	Triticum durum
Wheat, English	Pooideae	Triticum turgidum
Wheat, Large Spelt	Pooideae	Triticum spelta
Wheat, Polish	Pooideae	Triticum polonium
Wheat, Poulard	Pooideae	Triticum turgidum
Wheat, Singlegrained	Pooideae	Triticum monococcum
Wheat, Small Spelt	Pooideae	Triticum monococcum
Wheat, Soft	Pooideae	Triticum aestivum
Rice	Gramineae	Oryza sativa
Rice, American Wild	Gramineae	Zizania aquatica
Rice, Australian	Gramineae	Oryza australiensis
Rice, Indian	Gramineae	Zizania aquatica
Rice, Red	Gramineae	Oryza glaberrima
Rice, Tuscarora	Gramineae	Zizania aquatica
Rice, West African	Gramineae	Oryza glaberrima
Barley	Pooideae	Hordeum vulgare
Barley, Abyssinian Intermediate, also Irregular	Pooideae	Hordeum irregulare
Barley, Ancestral Tworow	Pooideae	Hordeum spontaneum
Barley. Beardless	Pooideae	Hordeum trifurcatum
Barley, Egyptian	Pooideae	Hordeum trifurcatum
Barley, fourrowed	Pooideae	Hordeum vulgare polystichon

COMMON NAME	FAMILY	LATIN NAME
Barley, sixrowed	Pooideae	Hordeum vulgare hexastichon
Barley, Tworowed	Pooideae	Hordeum distichon
Cotton, Abroma	Dicotyledoneae	Abroma augusta
Cotton, American Upland	Malvaceae	Gossypium hirsutum
Cotton, Asiatic Tree, also Indian Tree	Malvaceae	Gossypium arboreum
Cotton, Brazilian, also, Kidney, and, Pernambuco	Malvaceae	Gossypium barbadense brasiliense
Cotton, Levant	Malvaceae	Gossypium herbaceum
Cotton, Long Silk, also Long Staple, Sea Island	Malvaceae	Gossypium barbadense
Cotton, Mexican, also Short Staple	Malvaceae	Gossypium hirsutum
Soybean, Soya	Leguminosae	Glycine max
Sugar beet	Chenopodiaceae	Beta vulgaris altissima
Sugar cane	Woody-plant	Arenga pinnata
Tomato	Solanaceae	Lycopersicon esculentum
Tomato, Cherry	Solanaceae	Lycopersicon esculentum cerasiforme
Tomato, Common	Solanaceae	Lycopersicon esculentum commune
Tomato, Currant	Solanaceae	Lycopersicon pimpinellifolium
Tomato, Husk	Solanaceae	Physalis ixocarpa
Tomato, Hyenas	Solanaceae	Solanum incanum
Tomato, Pear	Solanaceae	Lycopersicon esculentum pyriforme
Tomato, Tree	Solanaceae	Cyphomandra betacea
Potato	Solanaceae	Solanum tuberosum
Potato, Spanish, Sweet potato	Convolvulaceae	Ipomoea batatas
Rye, Common	Pooideae	Secale cereale
Rye, Mountain	Pooideae	Secale montanum

COMMON NAME	FAMILY	LATIN NAME
Pepper, Bell	Solanaceae	Capsicum annuum grossum
Pepper, Bird, also Cayenne, Guinea	Solanaceae	Capsicum annuum minimum
Pepper, Bonnet	Solanaceae	Capsicum sinense
Pepper, Bullnose, also Sweet	Solanaceae	Capsicum annuum grossum
Pepper, Cherry	Solanaceae	Capsicum annuum cerasiforme
Pepper, Cluster, also Red Cluster	Solanaceae	Capsicum annuum fasciculatum
Pepper, Cone	Solanaceae	Capsicum annuum conoides
Pepper, Goat, also Spur	Solanaceae	Capsicum frutescens
Pepper, Long	Solanaceae	Capsicum frutescens longum
Pepper, Oranamental Red, also Wrinkled	Solanaceae	Capsicum annuum abbreviatum
Pepper, Tabasco Red	Solanaceae	Capsicum annuum conoides
Lettuce, Garden	Compositae	Lactuca sativa
Lettuce, Asparagus, also Celery	Compositae	Lactuca sativa asparagina
Lettuce, Blue	Compositae	Lactuca perennis
Lettuce, Blue, also Chicory	Compositae	Lactuca pulchella
Lettuce, Cabbage, also Head	Compositae	Lactuca sativa capitata
Lettuce, Cos, also Longleaf, Romaine	Compositae	Lactuca sativa longifolia
Lettuce, Crinkle, also Curled, Cutting, Leaf	Compositae	Lactuca sativa crispa
Celery	Umbelliferae	Apium graveolens dulce
Celery, Blanching, also Garden	Umbelliferae	Apium graveolens dulce
Celery, Root, also Turniprooted	Umbelliferae	Apium graveolens rapaceum
Eggplant, Garden	Solanaceae	Solanum melongena

COMMON NAME	FAMILY	LATIN NAME
Sorghum	Sorghum	All crop species
Alfalfa	Leguminosae	Medicago sativum
Carrot	Umbelliferae	Daucus carota sativa
Bean, Climbing	Leguminosae	Phaseolus vulgaris vulgaris
Bean, Sprouts	Leguminosae	Phaseolus aureus
Bean, Brazilian Broad	Leguminosae	Canavalia ensiformis
Bean, Broad	Leguminosae	Vicia faba
Bean, Common, also French, White, Kidney	Leguminosae	Phaseolus vulgaris
Bean, Egyptian	Leguminosae	Dolichos lablab
Bean, Long, also Yardlong	Leguminosae	Vigna sesquipedalis
Bean, Winged	Leguminosae	Psophocarpus tetragonolobus
Oat, also Common, Side, Tree	Avena	Sativa
Oat, Black, also Bristle, Lopsided	Avena	Strigosa
Oat, Bristle	Avena	
Pea, also Garden, Green, Shelling	Leguminosae	Pisum, sativum sativum
Pea, Blackeyed	Leguminosae	Vigna sinensis
Pea, Edible Podded	Leguminosae	Pisum sativum axiphium
Pea, Grey	Leguminosae	Pisum sativum speciosum
Pea, Winged	Leguminosae	Tetragonolobus purpureus
Pea, Wrinkled	Leguminosae	Pisum sativum medullare
Sunflower	Compositae	Helianthus annuus
Squash, Autumn, Winter	Dicotyledoneae	Cucurbita maxima
Squash, Bush, also Summer	Dicotyledoneae	Cucurbita pepo melopepo
Squash, Turban	Dicotyledoneae	Cucurbita maxima turbaniformis
Cucumber	Dicotyledoneae	Cucumis sativus

COMMON NAME	FAMILY	LATIN NAME
Cucumber, African, also Bitter		Momordica charantia
Cucumber, Squirting, also Wild		Ecballium elaterium
Cucumber, Wild		Cucumis anguria
Poplar, California	Woody-Plant	Populus trichocarpa
Poplar, European Black		Populus nigra
Poplar, Gray		Populus canescens
Poplar, Lombardy		Populus italica
Poplar, Silverleaf, also White		Populus alba
Poplar, Western Balsam		Populus trichocarpa
Tobacco	Solanaceae	Nicotiana
Arabidopsis Thaliana	Cruciferae	Arabidopsis thaliana
Turfgrass	Lolium	
Turfgrass	Agrostis	
	Other families of turfgrass	
Clover	Leguminosae	

Expression of the variant AHAS polypeptides in transgenic plants confers a high level of resistance to herbicides including, but not limited to, imidazolinone herbicides such as, for example, imazethapyr (PURSUIT©), allowing the use of these herbicides during cultivation of the transgenic plants.

Methods for the introduction of foreign genes into plants are known in the art. Non-limiting examples of such methods include *Agrobacterium* infection, particle bombardment, polyethylene glycol (PEG) treatment of protoplasts, electroporation of protoplasts, microinjection, macroinjection, tiller injection, pollen tube pathway, dry seed imbibition, laser perforation, and electrophoresis. These methods are described in, for example, B. Jenes et al., and S.W. Ritchie

et al. In *Transgenic Plants, Vol. 1, Engineering and Utilization*, ed. S.-D. Kung, R. Wu, Academic Press, Inc., Harcourt Brace Jovanovich 1993; and L. Mannonen et al., *Critical Reviews in Biotechnology*, 14:287-310, 1994.

In a preferred embodiment, the DNA encoding a variant AHAS is cloned into a DNA vector containing an antibiotic resistance marker gene, and the recombinant AHAS DNA-containing plasmid is introduced into *Agrobacterium tumefaciens* containing a Ti plasmid. This "binary vector system" is described in, for example, U.S. Patent No. 4, 490,838, and in An et al., *Plant Mol.Biol.Manual* A3:1-19 (1988). The transformed *Agrobacterium* is then co-cultivated with leaf disks from the recipient plant to allow infection and transformation of plant cells. Transformed plant cells are then cultivated in regeneration medium, which promotes the formation of shoots, first in the presence of the appropriate antibiotic to select for transformed cells, then in the presence of herbicide. In plant cells successfully transformed with DNA encoding herbicide-resistant AHAS, shoot formation occurs even in the presence of levels of herbicide that inhibit shoot formation from non-transformed cells. After confirming the presence of variant AHAS DNA using, for example, polymerase chain reaction (PCR) analysis, transformed plants are tested for their ability to withstand herbicide spraying and for their capabilities for seed germination and root initiation and proliferation in the presence of herbicide.

Other Applications

The methods and compositions of the present invention can be used in the structure-based rational design of herbicide-resistant AHAS variants, which can be incorporated into plants to confer selective herbicide resistance on the plants. Intermediate variants of AHAS (for example, variants that exhibit sub-optimal specific activity but high resistance and selectivity, or the converse) are useful as templates for the design of second-generation AHAS variants that retain adequate specific activity and high resistance and selectivity.

Herbicide resistant AHAS genes can be transformed into crop species in single or multiple copies to confer herbicide resistance. Genetic engineering of crop species with reduced sensitivity to herbicides can:

(1) Increase the spectrum and flexibility of application of specific effective and environmentally benign herbicides such as imidazolinone herbicides;

(2) Enhance the commercial value of these herbicides;

(3) Reduce weed pressure in crop fields by effective use of herbicides on herbicide resistant crop species and a corresponding increase in harvest yields;

(4) Increase sales of seed for herbicide-resistant plants;

(5) Increase resistance to crop damage from carry-over of herbicides applied in a previous planting;

(6) Decrease susceptibility to changes in herbicide characteristics due to adverse climate conditions; and

(7) Increase tolerance to unevenly or mis-applied herbicides.

For example, transgenic AHAS variant protein containing plants can be cultivated. The crop can be treated with a weed controlling effective amount of the herbicide to which the AHAS variant transgenic plant is resistant, resulting in weed control in the crop without detrimentally affecting the cultivated crop.

The DNA vectors described above that encode herbicide-resistant AHAS variants can be further utilized so that expression of the AHAS variant provides a selectable marker for transformation of cells by the vector. The intended recipient cells may be in culture or *in situ*, and the AHAS variant genes may be used alone or in combination with other selectable markers. The only requirement is that the recipient cell is sensitive to the cytotoxic effects of the cognate herbicide. This embodiment takes advantage of the relative low cost and lack of toxicity of, for example, imidazolinone-based herbicides, and may be applied in any system that requires DNA-mediated transformation.

Exemplification with respect to Preferred Embodiments

The following examples are intended to illustrate the present invention without limitation.

Example 1: Design of herbicide-resistant AHAS variants

Residues located close to the proposed herbicide binding site of the model described in detail above were selected for mutagenesis in order to design an active AHAS polypeptide with decreased herbicide binding capacity. Each site at the surface of the pocket was considered in terms of potential interactions with other residues in the pocket, as well as with cofactors and herbicides. For example, addition of positively charged residue(s) is expected to interfere with the charge distribution within the binding site, resulting in a loss in affinity of binding of a negatively-charged herbicide.

Three residues were identified as most useful targets for mutagenesis:

(1) F135 was believed to interact with both the isoalloxazine ring of FAD and with the aromatic group of the herbicides. In accordance with the strategy of introducing more charged residues into the binding pocket, this residue was changed to arginine.

(2) M53 contacts helix 498-507. This helix contains known herbicide resistance mutation sites and is also implicated in TPP binding. Furthermore, substitution of glutamic acid at position 53 was believed to favor an interaction with K185, reducing the affinity of K185 for the carboxylate group of imazethapyr.

(3) R128 is located near the entrance to the pocket, where it was believed to be involved in the initial transport of charged herbicides into the binding pocket. This residue was changed to alanine to remove both its charge and its long hydrophobic side chain.

Example 2: Site-directed mutagenesis of AHAS to produce herbicide-resistant variants

The *Arabidopsis* AHAS gene was inserted in-frame to the 3' end of the coding region of the glutathione S-transferase gene in the pGEX-2T vector (Pharmacia). Construction of the vector in this manner maintained the six amino acid thrombin recognition sequence at the junction of the expressed glutathione-S-transferase (GST)/AHAS fusion protein. Thrombin digestion of the expressed fusion protein results in an AHAS protein with an N-terminal starting position at the end of the transit peptide at a putative transit peptide processing site, with a residual N-terminal glycine derived from the thrombin recognition site. The final amino terminus of the cleaved AHAS protein consists of Gly-Ser-Ser-Ile-Ser. Site-directed mutations were introduced into the AHAS gene in this vector.

Site-directed mutations were constructed according to the PCR method of Higuchi (*Recombinant PCR*. In MA Innis, et al. *PCR Protocols: A Guide to Methods and Applications*, Academic Press, San Diego, pp. 177-183, 1990). Two PCR products, each of which overlap the mutation site, were amplified. The primers in the overlap region contained the mutation. The overlapping PCR amplified fragments were combined, denatured, and allowed to re-anneal together, producing two possible heteroduplex products with recessed 3'-ends. The recessed 3'-ends were extended by *Taq* DNA polymerase to produce a fragment that was the sum of the two overlapping PCR products containing the desired mutation. A subsequent re-amplification of this fragment with only the two "outside" primers resulted in the enrichment of the full-length product. The product containing the mutation was then re-introduced into the *Arabidopsis* AHAS gene in the pGEX-2T vector.

Example 3: Expression and Purification of AHAS Variants

A. Methods

E. Coli (DH5α) cells transformed with the pGEX-2T vector containing either the maize wild type AHAS gene (vector designation pAC751), the *Arabidopsis* Ser653Asn mutant, or the

Arabidopsis Ile401Phe mutant were grown overnight in LB broth containing 50 μg/mL ampicillin. The overnight culture of *E. coli* was diluted 1:10 in 1 L LB, 50 μg/mL ampicillin, and 0.1% v/v antifoam A. The culture was incubated at 37°C with shaking until the OD_{600} reached approximately 0.8. Isopropylthiogalactose (IPTG) was added to a final concentration of 1 mM and the culture was incubated for 3 more hours.

Cells were harvested by centrifugation at 8,670 xg for 10 minutes in a JA-10 rotor and resuspended in 1/100th of the original culture volume in MTPBS (16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 150 mM NaCl, pH 7.3). Triton X-100 and lysozyme were added to a final concentration of 1% v/v and 100 μg/mL, respectively. Cells were incubated at 30°C for 15 minutes cooled to 4°C on ice, and were lysed by sonication for 10 seconds at level 7 with a Branson Sonifier Cell Disrupter equipped with a microtip probe. The cell free extract was centrifuged at 35,000 xg for 10 min. at 4°C. The supernatant was decanted and the centrifugation step was repeated.

Purification of expressed fusion proteins was performed as modified from Smith and Johnson (Gene 67:31-40, 1988). The supernatant was warmed to room temperature and was passed through a 2 mL column of glutathione-agarose beads (sulfur linkage, Sigma) equilibrated in MTPBS. The column was subsequently washed with MTPBS at room temperature until the A_{280} of eluant matched that of MTPBS. The fusion protein was then eluted using a solution containing 5 mM reduced glutathione in 50 mM Tris HCL, pH 8.0. The eluted fusion protein was treated with approximately 30 NIH units of thrombin and dialyzed against 50 mM citrate pH 6.5 and 150 mM NaCl.

The fusion protein was digested overnight at room temperature. Digested samples were dialyzed against MTPBS and passed twice through a glutathione-agarose column equilibrated in MTPBS to remove the released glutathione transferase protein. The protein fraction that did not bind to the column was collected and was concentrated by ultrafiltration on a YM10 filter (Amicon). The concentrated sample was loaded onto a 1.5 x 95 cm Sephacryl S-100 gel filtration column equilibrated in

gel filtration buffer (50 mM HEPES, 150 mM NaCl, pH 7.0). Two mL fractions were collected at a flow rate of 0.14 mL/min. Enzyme stability was tested by storage of the enzyme at 4°C in gel filtration buffer with the addition of 0.02% sodium azide and in the presence or absence of 2 mM thiamine pyrophosphate and 100 μM flavin adenine dinucleotide (FAD).

B. Results

E. coli transformed with the pAC751 plasmid containing the wide-type AHAS gene fused downstream and in-frame with the GST gene expressed a 91 kD protein when induced with IPTG. The 91 kD protein exhibited the predicted molecular mass of a GST/AHAS fusion protein (the sum of 26 kD and 65 kD, respectively). When the cell free extract of DH5α /pAC751 was passed through a glutathione-agarose affinity gel, washed, and eluted with free glutathione it yielded a preparation enriched in the 91 kD protein (Figure 6, lane C). The six amino acid thrombin recognition site engineered in the junction of GST and AHAS was successfully cleaved by thrombin (Figure 6, lane D). The cleaved fusion protein preparation consisted of the expected 26 kD GST protein and the 65 kD maize AHAS protein. Maize AHAS was purified to homogeneity by a second pass through the glutathione-agarose column to affinity subtract GST and subjected to a final Sephacryl S-100 gel filtration step to eliminated thrombin (Figure 6, lane E). The 65 kD protein is recognized on western blots by a monoclonal antibody raised against a maize AHAS peptide.

Purified wild type maize AHAS was analyzed by electrospray mass spectrometry and was determined to have a molecular mass of 64,996 daltons (data not shown). The predicted mass, as calculated from the deduced amino acid sequence of the gene inserted into the pGEX-2T vector, is 65,058. The 0.096% discrepancy between the empirically determined and predicted mass was within tuning variability of the mass spectrometer. The close proximity of the two mass determinations suggests that there were no misincorporated nucleotides during construction of the expression vector, nor any post-translational modifications to the protein that would cause gross changes in molecular mass. Moreover, the lack of

spurious peaks in the preparation of purified enzyme indicated that the sample was free of contamination.

Example 4: Enzymatic properties of AHAS variants

The enzymatic properties of wild-type and variant AHAS produced in *E. coli* were measured by a modification of the method of Singh et al. (*Anal. Biochem* 171:173-179, 1988) as follows:

A reaction mixture containing 1X AHAS assay buffer (50 mM HEPES pH 7.0, 100 mM pyruvate, 10 mM $MgCl_2$, 1 mM thiamine pyrophosphate (TPP), and 50 μM flavin adenine dinucleotide (FAD)) was obtained either by dilution of enzyme in 2X assay buffer or by addition of concentrated enzyme to 1X AHAS assay buffer. All assays containing imazethapyr and associated controls contained a final concentration of 5% DMSO due to addition of imazethapyr to assay mixtures as a 50% DMSO solution. Assays were performed in a final volume of 250 μL at 37°C in microtiter plates. After allowing the reaction to proceed for 60 minutes, acetolactate accumulation was measured colorimetrically as described by Singh et al., *Anal. Biochem* 171:173-179, 1988.

Maize AHAS expressed and purified from pAC751 as described in Example 3 above is active in the conversion of pyruvate to acetolactate. Full AHAS activity is dependent on the presence of the cofactors FAD and TPP in the assay medium. No activity was detected when only FAD was added to the assay medium. The activity of the purified enzyme with TPP only, or with no cofactors, was less than 1% of the activity detected in the presence of both TPP and FAD. Normally, AHAS present in crude plant extracts is very labile, particularly in the absence of substrate and cofactors. In contrast, the purified AHAS from the bacterial expression system showed no loss in catalytic activity when stored for one month at 4°C in 50 mM HEPES pH 7.0, 150 mM NaCl, 0.02% NaN_3 in the presence or absence of FAD and TPP. Furthermore, no degradation products were visible from these stored preparations when resolved in SDS-PAGE gels.

The specific activities of wild-type AHAS and the M124E, R199A, and F206R variants are shown in Table 2 below. As determined from the alignment in Figure 5, the M124E mutation in *Arabidopsis* AHAS is the equivalent of the maize M53E mutation, the R199A mutation in *Arabidopsis* is the equivalent of the maize R128A mutation, and the F206R mutation in *Arabidopsis* is the equivalent of the maize F135R mutation. The mutations designed in the maize AHAS structural model were used to identify the equivalent amino acid in the dicot *Arabidopsis* AHAS gene and were incorporated and tested in the *Arabidopsis* AHAS gene. This translation and incorporation of rationally designed herbicide mutations into the dicot *Arabidopsis* AHAS gene can facilitate evaluation of herbicide resistance in plants of a dicot species.

TABLE 2

SPECIFIC ACTIVITY

	Specific Activity	% Catalytic Activity as Compared to Wild Type
Wild-Type	99.8	100
Met124Glu	9.15	9.16
Arg199Ala	86.3	86.5
Phe206Arg	5.07	5.1

The R199A mutation maintains a high level of catalytic activity (Table 2) while exhibiting a significant level of resistance to imazethapyr (Figure 7). Notably, this variant retains complete sensitivity to sulfonylureas (Figure 8). Thus, this variant fulfills the criteria of high specific activity and selective herbicide resistance. By contrast, the M124E substitution resulted in almost complete resistance to imazethapyr (Figure 7) but also exhibited severely reduced catalytic activity (Table 2). Relative to imidazolinone resistance, this variant exhibits greater sensitivity to sulfonylurea (Figure 8), suggesting that this residue is a good candidate for creating a mutation that confers selective resistance. Substitution of an amino acid other than glutamic acid may help to maintain catalytic activity. The F206R

substitution yielded similar results to those observed with M124E variant, but lacked selectivity in resistance.

Example 5: Iterative Improvement of AHAS Herbicide-Resistant Variant Using a Rational Design Approach

Changing residue 124 in AHAS from Met to Glu as described in Example 4 above conferred imidazolinone resistance but also reduced enzymatic activity to 9.2% of the wild type value. The model of the maize AHAS structure described above suggested that Met53 (equivalent to the *Arabidopsis* Met124 residue) interacts with a series of hydrophobic residues on the face of an α-helix that is derived from a separate subunit but are in close proximity to Met53. Thus, the hydrophobic interaction between Met53 and the residues on the helix may stabilize both subunit/subunit association and the conformation of the active site. It was believed that the substitution of the hydrophobic Met residue with a charged glutamate residue most probably destabilizes the inter-subunit hydrophobic interaction and results in a loss of catalytic activity.

Based on this structure/function analysis, the activity of the original *Arabidopsis* Met124Glu (equivalent to maize Met53Glu) mutant enzyme was then iteratively improved by substituting a more hydrophobic amino acid (Ile) at this position. The hydrophobic nature of the Ile side chain resulted in restoration of activity to wild type levels (specific activity of 102, equivalent to 102% of the wild-type activity), but the greater bulk of the Ile side chain was still able to maintain a significant level of imidazolinone resistance (Figure 9).

By comparison, substitution of a histidine residue at this position resulting in an AHAS variant exhibiting a specific activity of 42.5, equivalent to 42.6% of the wild-type activity. This mutant, nonetheless, exhibited a high degree of resistance to PURSUIT® (Figure 10).

Example 6: Iterative Improvement of AHAS Herbicide-Resistant Variant Using a Rational Design Approach

Another example of iterative refinement using the methods of the present invention involves the Arg128Ala variant. The structural model of maize AHAS suggested that the Arg128 residue, which resides at the lip of the herbicide binding pocket, contributes to channeling charged substrates and herbicides into the herbicide binding pocket and into the active site. The Arg 128 residue is distant from the TPP moiety, which binds the initial pyruvate molecule in the reaction mechanism of AHAS, explaining why the substitution of *Arabidopsis* AHAS Arg199 (the equivalent to maize Arg128) to alanine had little effect on the catalytic activity of the enzyme. The structural model further indicated that a more radical change could be made at this position to raise the level of resistance while maintaining high levels of catalytic activity. On this basis, an iterative improvement of the mutation was made to substitute the positively charge arginine residue with a negatively charged glutamate residue. The enzyme thus mutated had improved levels of resistance to PURSUIT® while maintaining high levels of activity (specific activity of 114, equivalent to 114% of the wild-type activity) (Figure 11).

Example 7: Interchangeability of AHAS Derived From Different Species in Structure-Based Rational Design of Herbicide-Resistant AHAS Variants

A structural model of the three-dimensional structure of AHAS is built with a monocot AHAS sequence such as that derived from maize, as described above. To introduce mutations into AHAS derived from a dicot species such as *Arabidopsis*, the sequences of AHAS derived from the monocot and dicot species are aligned using the GAP and PILEUP programs (Genetics Computer Group, 575 Sequence Drive, Madison, WI 53711). Equivalent positions are determined from the computer-generated alignment. The mutations are then introduced into the dicot AHAS gene as described above. Following expression of the mutant AHAS protein in *E. coli* and assessment of its

biochemical properties (i.e., specific activity and resistance to herbicides), the mutant gene is introduced into a dicot plant by plant transformation methods as described above.

Example 8: Production of Herbicide-Resistant Plants by Transformation with Rationally Designed AHAS Genes

DNA Constructs:

Rationally designed AHAS variant genes contained within *E. coli* expression vectors were used as a source of DNA restriction fragments to replace the equivalent restriction fragment in a *Arabidopsis* AHAS gene. This gene is present in a 5.5 kb genomic DNA fragment which also contains the *Arabidopsis* AHAS promoter, the *Arabidopsis* AHAS termination sequence and 5'- and 3'-flanking DNA. After DNA sequencing through the mutation sites was performed to confirm the presence of the proper mutation, the entire 5.5 kb fragment from each plasmid was inserted into a pBIN based plant transformation vector (Mogen, Leiden, Netherlands). The plant transformation vector also contains the neomycin phosphotransferase II (nptII) kanamycin resistance gene driven by the 35S cauliflower mosaic virus promoter. The final vector construct is displayed in Figure 12. Vectors containing *Arabidopsis* AHAS genes with Met124Ile, Met124His, and Arg199Glu mutations (corresponding to Met53Ile, Met53His, and Arg128Glu mutations in the maize AHAS sequence as shown in Figure 1) were labeled pJK002, pJK003, and pJK004, respectively.

Each of these vectors was transformed into *Agrobacterium tumefaciens* strain LBA4404 (R&D Life Technologies, Gaithersburg, MD) using the transformation method described in An et al., *Plant Mol.Biol.Manual* A3:1-19 (1988).

Plant Transformation:

Leaf disc transformation of *Nicotiana tabacum* cv. Wisconsin 38 was performed as described by Horsch et al. (*Science*, 227: 1229-1231, 1985) with slight modifications. Leaf discs were cut from plants grown under sterile conditions and co-cultivated upsidedown in Murashige Skoog media (Sigma Chemical Co., St. Louis, MO) for 2-3 days at 25°C in darkness

with *Agrobacterium tumefaciens* strains containing plasmids pJK002, pJK003, or pJK004. The discs were blotted dry and transferred to regeneration Murashige Skoog medium with B5 vitamins containing 1 mg/L benzyladenine and 0.1 mg/l 1-Napthyl Acetic Acid, 100 mg/L kanamycin, and 500 mg/L cefotaxime (all obtained from Sigma).

Initially, transformants were selected by kanamycin resistance conferred by the nptII gene present in the transformation vector. Shoots derived from the leaf discs were excised and placed on fresh Murashige Skoog hormone free media containing cefotaxime and kanamycin.

In Vivo Herbicide Resistance

Kanamycin-resistant tobacco shoots were transferred to medium containing a 0.25 μM imazethapyr. At this concentration of the imidazolinone herbicide, non-transformed tobacco shoots (containing endogenous wild-type AHAS) were not able to initiate root formation. By contrast, root initiation and growth were observed from tobacco shoots transformed with each of the mutant AHAS genes. Roots developed from shoots transformed with the Met124Ile and Arg199Glu mutant genes along with wild type are shown in Figure 1. Furthermore, plants transformed with the Met124Ile or Arg199Glu mutant genes were resistant to spraying with twice the field rate (100 g/ha) of imazethapyr (Figure 13). The patterns of root growth in transformed vs. non-transformed plants in the presence of herbicide, as well as the behavior after herbicide spraying suggest that expression of the rationally designed herbicide resistance genes confers herbicide resistance *in vivo*.

Detection of the Rationally Designed Genes in Herbicide Resistant Tobacco

Genomic DNA was isolated from the AHAS-transformed tobacco plants, and the presence of the *Arabidopsis* AHAS variant genes was verified by PCR analysis. Differences between the nucleotide sequences of the *Arabidopsis* AHAS gene and the two tobacco AHAS genes were exploited to design PCR primers that amplify only the *Arabidopsis* gene in a tobacco genomic DNA background. The rationally designed herbicide resistance genes were detected, as shown by amplification of

a DNA fragment of the proper size, in a majority of the herbicide resistant plants. No PCR signal was seen from non-transformed tobacco plants.

Segregation of Transformed AHAS Genes:

To monitor segregation of rationally designed AHAS genes in transformed plants, germination tests were performed. Seeds were placed in hormone-free Murashige-Skoog medium containing up to 2.5 μM PURSUIT© and 100 μM kanamycin. The seedlings that resulted were visually scored for resistance or susceptibility to the herbicide.

Since tobacco plants are diploid, it would be expected that the progeny of self-pollinated plants should segregate 3:1 resistant:susceptible, reflecting the existence of 1 seedling homozygous for the resistant AHAS gene, 2 seedlings heterozygous for the resistant AHAS gene, and 1 seedling lacking a resistant AHAS gene.

The results indicate that resistant AHAS genes are segregating in the expected 3:1 ratio, supporting the conclusion that herbicide resistance is conferred by a single, dominant copy of a rationally designed AHAS gene.

These results indicate that rational design of herbicide-resistant AHAS genes can be used to produce plants that exhibit herbicide resistant growth *in vivo*.

Example 9: Production of Plants Cross-Resistant to Different Herbicides by Transformation with Rationally Designed AHAS Genes

Tobacco plants transformed with rationally designed AHAS genes as described in Example 8 above were also tested for cross-resistance to another herbicide, CL 299,263 (also known as imazamox). Germination tests were performed on seeds harvested from the primary transformants containing the Met124Ile, Met124His, and Arg199Glu *Arabidopsis* AHAS variant genes, in the absence or presence of 2.5 μM CL 299,263 (Figure 15). This concentration of the herbicide causes severe stunting and bleaching of wild-type tobacco plants. Tobacco plants transformed with the Met124His AHAS gene showed the greatest level of resistance (Figure 15). Arg199Glu

transformants showed an intermediate level of resistance, while Met124Ile showed little resistance (Figure 15).

All patents, applications, articles, publications, and test methods mentioned above are hereby incorporated by reference.

Many variations of the present invention will suggest themselves to those skilled in the art in light of the above detailed description. Such obvious variations are within the full intended scope of the appended claims.

WHAT IS CLAIMED IS

1. A structure-based modelling method for the production of an herbicide resistant AHAS variant protein, said method comprising:

(a) aligning a target AHAS protein on pyruvate oxidase template or an AHAS modelling equivalent thereof to derive the three-dimensional structure of said target AHAS protein;

(b) modelling one or more herbicides into said three-dimensional structure to localize an herbicide binding pocket in said target AHAS protein;

(c) selecting as a target for a mutation an amino acid position in said target AHAS protein, wherein said mutation alters the affinity of at least one herbicide for said binding pocket;

(d) mutating DNA encoding said target AHAS protein to produce a mutated DNA encoding a variant AHAS containing said mutation at said position; and

(e) expressing said mutated DNA in a first cell, under conditions in which said variant AHAS containing said mutation at said position is produced.

2. A structure-based modelling method as defined in claim 1, further comprising:

(f) expressing DNA encoding wild-type AHAS in parallel in a second cell;

(g) purifying said wild-type and said variant AHAS proteins from said cells;

(h) assaying said wild-type and said variant AHAS proteins for catalytic activity in the conversion of pyruvate to acetolactate or in the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate, in the absence and in the presence of said at least one herbicide; and

(i) repeating steps (c)-(h), wherein said DNA encoding said variant of step (e) is used as the AHAS-encoding DNA in step (c) until a first herbicide resistant AHAS variant protein is identified having:

(i) in the absence of said at least one herbicide,

(a) a catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

(b) catalytic activity in combination with any herbicide resistant AHAS variant protein also expressed in said cell, which may be the same as or different than said first AHAS variant protein, sufficient to maintain the viability of a cell in which it is expressed;

wherein said cell requires AHAS activity for viability; and

(ii) catalytic activity that is more resistant to at least one herbicide than is wild type AHAS.

3. A structure-based modelling method as defined in claim 1, wherein said AHAS modelling equivalent is selected from the group consisting of transketolases, carboligases, and pyruvate decarboxylase.

4. A structure-based modelling method as defined in claim 2, wherein said catalytic activity in the absence of said more than one herbicide is more than about 20% of the catalytic activity of said wild-type AHAS in the above of said at least one herbicide.

5. A structure-based modelling method as defined in claim 4, wherein said herbicide is an imidazolinone herbicide and said first herbicide-resistant AHAS variant protein has:

(i) catalytic activity in the absence of said herbicide of more than about 20% of the catalytic activity of said wild-type AHAS;

(ii) catalytic activity that is relatively more resistant to the presence of imidazolinone herbicides compared to wild type AHAS; and

(iii) catalytic activity that is relatively more sensitive to the presence of sulfonylurea herbicides compared to imidazolinone herbicides.

6. A structure-based modelling method as defined in claim 1, wherein said herbicides are selected from the group consisting of imidazolinones, sulfonylureas, triazolopyrimidine sulfonamides, pyrimidyl-oxy-benzoic acids, sulfamoylureas sulfocarboxamides, and combinations thereof.

7. A structure-based modelling method as defined in claim 1, wherein said target AHAS protein is derived from *Arabidopsis thaliana*.

8. A structure-based modelling method as defined in claim 1, wherein said first cell is *E. coli*.

9. A structure-based modelling method as defined in claim 2, wherein said first and second cells are *E. coli*.

10. A structure-based modelling method as defined in claim 1, wherein said target AHAS protein comprises a protein having the sequence of Figure 1 or a functional equivalent thereof.

11. A structure-based modelling method as defined in claim 1, wherein said mutation is selected from the group consisting of

(i) substitution of at least one different amino acid residue at an amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311,

E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, and G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing ;

(ii) deletion of up to 5 amino acid residues preceding, or up to 5 amino acid residues following at least one amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G 583, and G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing ;

(iii) deletion of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(iv) addition of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(v) deletion of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1;

(vi) addition of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1; or

(vii) any combination of any of the foregoing.

12. A structure-based modelling method as defined in claim 11, wherein said substitution is selected from the group consisting of Met53Trp, Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg, Ile330Phe, a functional equivalent of any of the foregoing, or a combination of any of the foregoing.

13. A structure-based modelling method for the production of herbicide resistant AHAS variant protein, said method comprising:

(a) aligning a target AHAS protein on a first AHAS template derived from a polypeptide having the sequence of Figure 1 or a functional equivalent thereof, to derive the three-dimensional structure of said target AHAS protein;

(b) modelling one or more herbicides into said three-dimensional structure to localize an herbicide binding pocket in said target AHAS protein;

(c) selecting as a target for a mutation, at least one amino acid position in said target AHAS protein, wherein said mutation alters the affinity of at least one herbicide for said binding pocket;

(d) mutating DNA encoding said target AHAS protein to produce a mutated DNA encoding a variant AHAS containing the mutation at said position; and

(e) expressing said mutated DNA in a first cell, under conditions in which said variant AHAS containing said mutation at said position is produced.

14. A structure-based modelling method as defined in claim 13, further comprising:

(f) expressing DNA encoding wild-type AHAS in parallel in a second cell;

(g) purifying said wild-type and said variant AHAS proteins from said cells;

(h) assaying said wild-type and said variant AHAS proteins for catalytic activity in conversion of pyruvate to acetolactate or in the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate, in the absence and in the presence of said at least one herbicide; and

(i) repeating steps (c)-(h), wherein said DNA encoding the variant of step (e) is used as the AHAS-encoding DNA in step (c) until a first herbicide resistant AHAS variant protein is identified having:

(i) in the absence of said at least one herbicide,

(a) a catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

(b) catalytic activity in combination with any herbicide resistant AHAS variant protein also expressed in said cell, which may be the same as or different than said first AHAS variant protein, sufficient to maintain the viability of a cell in which it is expressed;

wherein said cell requires AHAS activity for viability; and

(ii) catalytic activity that is more resistant to at least one herbicide than is wild type AHAS.

15. A structure-based_modelling method as defined in claim 14, wherein said catalytic activity in the absence of said at least one herbicide is more than about 20% of the catalytic activity of said wild-type AHAS.

16. A structure-based modelling method as defined in claim 15, wherein said herbicide is an imidazolinone herbicide and said first herbicide resistant AHAS variant protein has:

(i) catalytic activity in the absence of said herbicide of more than about 20% of the catalytic activity of said wild-type AHAS;

(ii) catalytic activity that is relatively more resistant to the presence of imidazolinone herbicides compared to wild type AHAS; and

(iii) catalytic activity that is relatively more sensitive to the presence of sulfonylurea herbicides compared to imidazolinone herbicides.

17. A structure-based modelling method as defined in claim 13, wherein said herbicide is selected from the group consisting of imidazolinones, sulfonylureas, triazolopyrimidine sulfonamides, pyrimidyl-oxy-benzoic acids, sulfamoylureas, sulfonylcarboxamides, and combinations thereof.

18. A structure-based modelling method as defined in claim 13, wherein said target AHAS protein is derived from *Arabidopsis thaliana*.

19. A structure-based modelling method as defined in claim 13, wherein said first cell is *E. coli*.

20. A structure-based modelling method as defined in claim 19, wherein said first and second cells are *E. coli*.

21. A structure-based modelling method as defined in claim 13, wherein said mutation is selected from the group consisting of

(i) substitution of at least one different amino acid residue at an amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301,

R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing;

(ii) deletion of up to 5 amino acid residues preceding, or up to 5 amino acid residues following at least one amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing;

(iii) deletion of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(iv) addition of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(v) deletion of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1;

(vi) addition of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1; or

(vii) any combination of any of the foregoing.

22. A structure-based modelling method as defined in claim 21, wherein said substitution is selected from the group consisting of Met53Trp, Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg, Ile330Phe, a functional equivalent of any of the foregoing, or a combination of any of the foregoing.

23. A structure-based modelling method for the production of herbicide resistant AHAS variant protein, said method comprising:

(a) aligning a target AHAS protein on a first AHAS template having an identified herbicide binding pocket and having the sequence of Figure 1 or a functional equivalent thereof, to derive the three-dimensional structure of said target AHAS protein;

(b) selecting as a target for a mutation, at least one amino acid position in said target AHAS protein, wherein said mutation alters the affinity of at least one herbicide for said binding pocket;

(c) mutating DNA encoding said target AHAS protein to produce a mutated DNA encoding a variant AHAS containing said mutation at said position; and

(d) expressing said mutated DNA in a first cell, under conditions in which said variant AHAS containing said mutation at said position is produced.

24. A structure-based modelling method as defined in claim 23, further comprising:

(e) expressing DNA encoding wild-type AHAS in parallel in a second cell;

(f) purifying said wild-type and said variant AHAS proteins from said cells;

(g) assaying said wild-type and said variant AHAS proteins for catalytic activity in conversion of pyruvate to acetolactate or in the condensation of pyruvate and 2-ketobutyrate to form acetohydroxybutyrate, in the absence and in the presence of said at least one herbicide; and

(h) repeating steps (b)-(g), wherein said DNA encoding said AHAS of step (d) is used as the AHAS-encoding DNA in step (b) until a first herbicide resistant AHAS variant protein is identified having:

(i) in the absence of said at least one herbicide,

(a) a catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

(b) catalytic activity in combination with any herbicide resistant AHAS variant protein also expressed in said cell, which may be the same as or different than said first AHAS variant protein, sufficient to maintain the viability of a cell in which it is expressed;

wherein said cell requires AHAS activity for viability; and

(ii) catalytic activity that is more resistant to at least one herbicide than is wild type AHAS.

25. A structure-based modelling method as defined in claim 24, wherein said catalytic activity in the absence of said at least one herbicide is more than about 20% of the catalytic activity of said wild-type AHAS.

26. A structure-based modelling method as defined in claim 25, wherein said herbicide is an imidazolinone herbicide and a first herbicide resistant AHAS variant protein has:

(i) catalytic activity in the absence of said herbicide of more than about 20% of the catalytic activity of said wild-type AHAS;

(ii) catalytic activity that is relatively more resistant to the presence of imidazolinone herbicides compared to wild type AHAS; and

(iii) catalytic activity that is relatively more sensitive to the presence of sulfonylurea herbicides compared to imidazolinone herbicides.

27. A structure-based modelling method as defined in claim 23, wherein said herbicide is selected from the group consisting of imidazolinones, sulfonylureas, triazolopyrimidine sulfonamides, pyrimidyl-oxy-benzoic acids, sulfamoylureas, sulfonylcarboxamides, and combinations thereof.

28. A structure-based modelling method as defined in claim 23, wherein said target AHAS protein is derived from *Arabidopsis thaliana*.

29. A structure-based modelling method as defined in claim 23, wherein said cell is *E. coli*.

30. A structure-based modelling method as defined in claim 24, wherein said first and second cells are *E. coli*.

31. A structure-based modelling method as defined in claim 23, wherein said mutation is selected from the group consisting of

(i) substitution of at least one different amino acid residue at an amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301,

R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing;

(ii) deletion of up to 5 amino acid residues preceding, or up to 5 amino acid residues following at least one amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing;

(iii) deletion of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(iv) addition of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(v) deletion of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1;

(vi) addition of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1; or

(vii) any combination of any of the foregoing.

32. A structure-based modelling method as defined in claim 31, wherein said substitution is selected from the group consisting of Met53Trp, Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg, Ile330Phe, a functional equivalent of any of the foregoing, or a combination of any of the foregoing.

33. An isolated DNA encoding an acetohydroxy acid synthase (AHAS) variant protein, said variant protein comprising an AHAS protein modified by

(i) substitution of at least one different amino acid residue at an amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing;

(ii) deletion of up to 5 amino acid residues preceding, or up to 5 amino acid residues following at

least one amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing;

(iii) deletion of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(iv) addition of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(v) deletion of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1;

(vi) addition of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1; or

(vii) any combination of any of the foregoing, or functional equivalents thereof.

34. DNA as defined in claim 33, wherein said modification alters the ability of an herbicide to inhibit the enzymatic activity of said protein.

35. DNA as defined in claim 34, wherein said herbicide is selected from the group consisting of

imidazolinones, sulfonylureas, triazolopyrimidine sulfonamides, pyrimidyl-oxy-benzoic acids, sulfamoylureas, sulfonylcarboxamides, and combinations thereof.

36. DNA as defined in claim 33, wherein said AHAS protein is derived from *Arabidopsis thaliana*.

37. DNA as defined in claim 33, wherein said substitution is selected from the group consisting of Met53Trp, Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg, Ile330Phe, a functional equivalent of any of the foregoing, or a combination of any of the foregoing.

38. DNA as defined in claim 37, wherein said variant AHAS protein has

(a) in the absence of at least one AHAS inhibiting herbicide,

(i) a catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

(ii) catalytic activity in combination with any second herbicide resistant AHAS variant protein also expressed in said cell, which may be the same as or different than said AHAS variant protein, sufficient to maintain the viability of a cell in which it is expressed;

wherein said cell requires AHAS activity for viability; and

(b) catalytic activity that is more resistant to at least one herbicide than is wild type AHAS.

39. DNA as defined in claim 33, wherein said variant AHAS has more than about 20% of the catalytic activity of wild-type AHAS.

40. DNA defined in claim 39, wherein said variant AHAS is at least 2-fold more resistant to imidazolinone-based herbicides than to sulfonylurea-based herbicides.

41. A DNA vector comprising the DNA sequence of claim 33 operably linked to a transcription regulatory element.

42. A cell comprising an AHAS encoding DNA sequence derived from a DNA vector as defined in claim 41, wherein said cell is selected from the group consisting of bacterial, fungal, plant, insect, and mammalian cells.

43. A cell as defined in claim 42, comprising a plant cell.

44. A seed comprising a cell as defined in claim 43.

45. A variant AHAS protein comprising a protein encoded by a DNA as defined in claim 33.

46. A variant AHAS protein comprising an AHAS protein modified by

(i) substitution of at least one different amino acid residue at an amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96 ,S97 ,G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508,

K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing;

(ii) deletion of up to 5 amino acid residues preceding, or up to 5 amino acid residues following at least one amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing;

(iii) deletion of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(iv) addition of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(v) deletion of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1;

(vi) addition of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1; or

(vii) any combination of any of the foregoing.

47. A variant AHAS protein as defined in claim 46, wherein said modification alters the ability of a herbicide to inhibit the enzymatic activity of said protein.

48. A variant AHAS protein as defined in claim 46, wherein said herbicide is selected from the group consisting of an imidazolinones, sulfonylureas, triazolopyrimidine, sulfomamides, pyrimidyl-oxy-benzoic acids, sulfamoylureas, sulfonylcarboxamides, and combinations thereof.

49. A variant AHAS protein as defined in claim 46, wherein said AHAS protein is derived from *Arabidopsis thaliana*.

50. A variant AHAS protein as defined in claim 46, wherein said substitution is selected from the group consisting of Met53Trp, Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg, Ile330Phe, a functional equivalent of any of the foregoing, or a combination of any of the foregoing or functional equivalents thereof.

51. A variant AHAS protein as defined in claim 46, wherein said variant AHAS protein has

(a) in the absence of said at least one AHAS inhibiting herbicide,

(i) a catalytic activity alone sufficient to maintain the viability of a cell in which it is expressed; or

(ii) catalytic activity in combination with any second herbicide resistant AHAS variant protein also expressed in said cell, which may be the same as or different than said AHAS variant protein, sufficient to maintain the viability of a cell in which it is expressed;

wherein said cell requires AHAS activity for viability; and

(b) catalytic activity that is more resistant to at least one herbicide than is wild type AHAS.

52. A variant AHAS protein as defined in claim 46, wherein said variant AHAS has more than about 20% of the catalytic activity of wild-type AHAS.

53. A method for conferring herbicide resistance on a cell, said method comprising:

(a) cloning a DNA as defined in claim 33 into a compatible expression vector; and

(b) transforming said DNA into said cell, under conditions wherein said gene is expressed at sufficient levels to confer herbicide resistance on said cell.

54. A cell prepared according to the method of claim 53.

55. A plant comprising a cell as defined in claim 54.

56. A method as defined in claim 53, wherein said mutated gene encodes a different amino acid at least one of positions 53, 128, 135, or combinations thereof.

57. A method as defined in claim 56, wherein said AHAS gene comprises the *Arabidopsis thaliana* AHAS gene.

58. A method as defined in claim 53, wherein said cell is selected from the group consisting of bacterial, fungal, plant, insect, and mammalian cells.

59. A method as defined in claim 58, wherein said cell is a plant cell.

60. A method as defined in claim 59, wherein said cell is in a seed.

61. A method for production of herbicide-resistant AHAS protein, said method comprising:

(a) selecting an amino acid position in an AHAS protein as a target for mutation;

(b) mutating DNA encoding AHAS to produce a mutated DNA encoding a mutation at said position;

(c) expressing said mutated DNA in a first cell, under conditions in which a variant AHAS is produced containing said mutation at said position;

(d) expressing wild-type AHAS protein in parallel in a second cell;

(e) purifying said wild-type and said variant AHAS proteins, from said cells;

(f) assaying said wild-type and said variant AHAS proteins, for catalytic activity in conversion of pyruvate to acetolactate, in the absence and in the presence of imidazolinone or sulfonylurea herbicides; and

(g) repeating steps (a)-(g), wherein said mutated DNA is used as the AHAS-encoding DNA in step (b) until an herbicide resistant AHAS protein having:

(i) catalytic activity in the absence of herbicides of more than about 20% of the catalytic activity of said wild-type AHAS;

(ii) catalytic activity that is relatively more resistant to the presence of imidazolinone herbicides compared to wild type AHAS; and

(iii) catalytic activity that is relatively more sensitive to the presence of sulfonylurea herbicides compared to imidazolinone herbicides is identified.

62. A method as defined in claim 61, wherein said herbicide is selected from the group consisting of imidazolinones, sulfonylureas, triazolopyrimidine sulfonamides, pyrimidyl-oxy-benzoic acids,

sulfamoylureas, sulfonylcarboxamides, and combinations thereof.

63. A method as defined in claim 61, wherein said AHAS protein is derived from *Arabidopsis thaliana*.

64. A method as defined in claim 61, wherein said cell is *E. coli*.

65. A method as defined in claim 61, wherein said target AHAS protein comprises a protein having the sequence of Figure 1.

66. A method as defined in claim 65, wherein said mutation is selected from the group consisting of

(i) substitution of at least one different amino acid residue at an amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125, R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing;

(ii) deletion of up to 5 amino acid residues preceding, or up to 5 amino acid residues following at least one amino acid residue of the sequence of Figure 1 selected from the group consisting of P48, G49, S52, M53, E54, A84, A95, T96, S97, G98, P99, G100, A101, V125,

R127, R128, M129, I130, G131, T132, D133, F135, Q136, D186, I187, T259, T260, L261, M262, G263, R276, M277, L278, G279, H281, G282, T283, V284, G300, V301, R302, F303, D304, R306, V307, T308, G309, K310, I311, E312, A313, F314, A315, S316, R317, A318, K319, I320, E329, I330, K332, N333, K334, Q335, T404, G413, V414, G415, Q416, H417, Q418, M419, W420, A421, A422, L434, S435, S436, A437, G438, L439, G440, A441, M442, G443, D467, G468, S469, L471, N473, L477, M479, Q495, H496, L497, G498, M499, V501, Q502, Q504, D505, R506, Y508, K509, A510, N511, R512, A513, H514, T515, S524, H572, Q573, E574, H575, V576, L577, P578, M579, I580, P581, G583, G584, functional equivalents of any of the foregoing, and any combination of any of the foregoing;

(iii) deletion of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(iv) addition of at least one amino acid residue or a functional equivalent thereof between Q124 and H150 of the sequence of Figure 1;

(v) deletion of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1;

(vi) addition of at least one amino acid residue or a functional equivalent thereof between G300 and D324 of the sequence of Figure 1; or

(vii) any combination of any of the foregoing.

67. A method as defined in claim 66, wherein said substitution is selected from the group consisting of Met53Trp, Met53Glu, Met53Ile, Met53His, Arg128Ala, Arg128Glu, Phe135Arg, Ile330Phe, a functional equivalent of any of the foregoing, or a combination of any of the foregoing.

68. A method for controlling weeds in a crop, said method comprising cultivating a crop comprising herbicide resistant plants as defined in claim 55, and treating

said crop with a weed controlling effective amount of said herbicide.

69. A method for controlling weeds in a crop, said method comprising cultivating a crop comprising herbicide resistant plants as defined in claim 55, and treating said crop with a weed controlling effective amount of an herbicidal composition comprising said herbicide.

70. A structure-based modelling method for the preparation of a first herbicide which inhibits AHAS activity, said method comprising:

(a) aligning a target AHAS protein on pyruvate oxidase template or an AHAS modelling functional equivalent thereof to derive the three-dimensional structure of said target AHAS protein;

(b) modelling a second herbicide having AHAS inhibiting activity into said three-dimensional structure to derive the location, structure, or a combination thereof of an herbicide binding pocket in said target AHAS protein; and

(c) designing a non-peptidic first herbicide which will interact with an AHAS activity inhibiting effective portion of said binding pocket, wherein said first herbicide inhibits said AHAS activity sufficiently to destroy the viability of a cell which requires AHAS activity for viability.

71. A structure-based modelling method as defined in claim 70, wherein said AHAS modelling functional equivalent is selected from the group consisting of transketolases, carboligases, and pyruvate decarboxylase.

72. A structure-based modelling method as defined in claim 70, wherein said first herbicide contains at least one functional group that interacts with a functional group of said binding pocket.

73. A structure-based modelling method as defined in claim 70, wherein said first herbicide binds to at least an AHAS activity-inhibiting effective portion of said binding pocket.

74. A structure-based modelling method as defined in claim 70, wherein said first herbicide inhibits said catalytic activity of said AHAS to less than about 20% of the catalytic activity of wild-type AHAS.

75. A structure-based modelling method as defined in claim 70, wherein said target AHAS protein is derived from *Arabidopsis thaliana*.

76. A structure-based modelling method as defined in claim 70, wherein said target AHAS protein comprises a protein having the sequence of Figure 1 or a functional equivalent thereof.

77. A structure-based modelling method for the production of a first herbicide which inhibits AHAS activity, said method comprising:

(a) aligning a target AHAS protein on a first AHAS template derived from a polypeptide having the sequence of Figure 1 or a functional equivalent thereof, to derive the three-dimensional structure of said target AHAS protein;

(b) modelling a second herbicide having AHAS inhibiting activity into said three-dimensional structure to derive the location, structure, or a combination thereof of an herbicide binding pocket in said target AHAS protein; and

(c) designing a non-peptidic first herbicide which will interact with an AHAS activity inhibiting effective portion of said binding pocket, wherein said first herbicide inhibits said AHAS activity sufficiently to destroy the viability of a cell which requires AHAS activity for viability.

78. A structure-based modelling method as defined in claim 77, wherein said first herbicide contains at least one functional group that interacts with a functional group of said binding pocket.

79. A structure-based modelling method as defined in claim 77, wherein said first herbicide binds to at least an AHAS activity inhibiting effective portion of said binding pocket.

80. A structure-based modelling method as defined in claim 77, wherein said first herbicide inhibits said catalytic activity of said AHAS to less than about 20% of the catalytic activity of wild-type AHAS.

81. A structure-based modelling method as defined in claim 77, wherein said target AHAS protein is derived from *Arabidopsis thaliana*.

82. A structure-based modelling method for the production of a first herbicide which inhibits AHAS activity, said method comprising:

(a) designing a non-peptide herbicide which will interact with an AHAS activity inhibiting-effective portion of a binding pocket of an AHAS protein, wherein said herbicide inhibits said AHAS activity sufficiently to destroy the viability of a cell which requires AHAS activity for viability.

83. A cell transformed with a DNA as defined in claim 33, wherein said DNA is expressed in said cell at sufficient levels to confer herbicide resistance on said cell.

84. A plant transformed with a DNA as defined in claim 33, wherein said DNA is expressed in said plant at sufficient levels to confer herbicide resistance on said plant.

FIG. 1a

*1 * GSAASPAMP*10 *MAPPATPLRP*20 *WGPTDPRKGA*

*60 *TRSPVIANHL*70 *FRHEQGEAFA*80 *ASGYARSSGR*

Arg128 Phe135

*120*AITGQVPRRM*130*IGTDAFQETP*140*IVEVTRSITK*

*180*LVDIPKDIQQ*190*QMAVPVWDKP*200*MSLPGYIARL*

*240*ARSGEELRRF*250*VELTGIPVTT*260*TLMGLGNFPS*

*300*GVRFDDRVTG*310*KIEAFASRAK*320*IVIIVDIDPAE*

*360*SKKSFDFGSW*370*NDELDQQKRE*380*FPLGYKTSNE*

*420*WAAQYYTYKR*430*PRQWLSSAGL*440*GAMGFGLPAA*

*480*IRIENLPVKV*490*FVLNNQHLGM*500*VVQWEDRFYK*

*540*PAVRVTKKNE*550*VRAAIKKMLE*560*TPGPYLLDII*

A

A

FIG. 1b

FIG. 2a

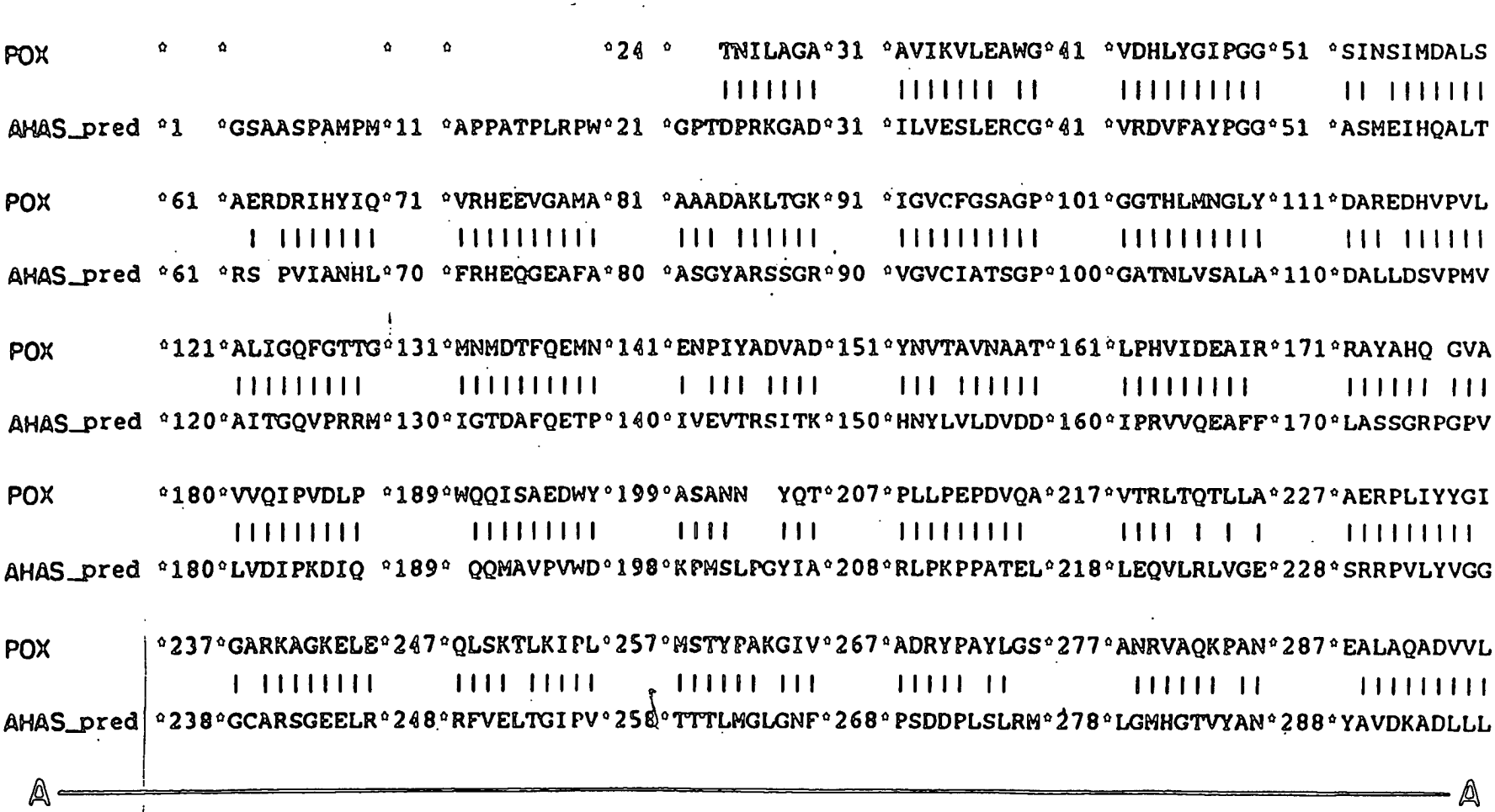


FIG. 2b

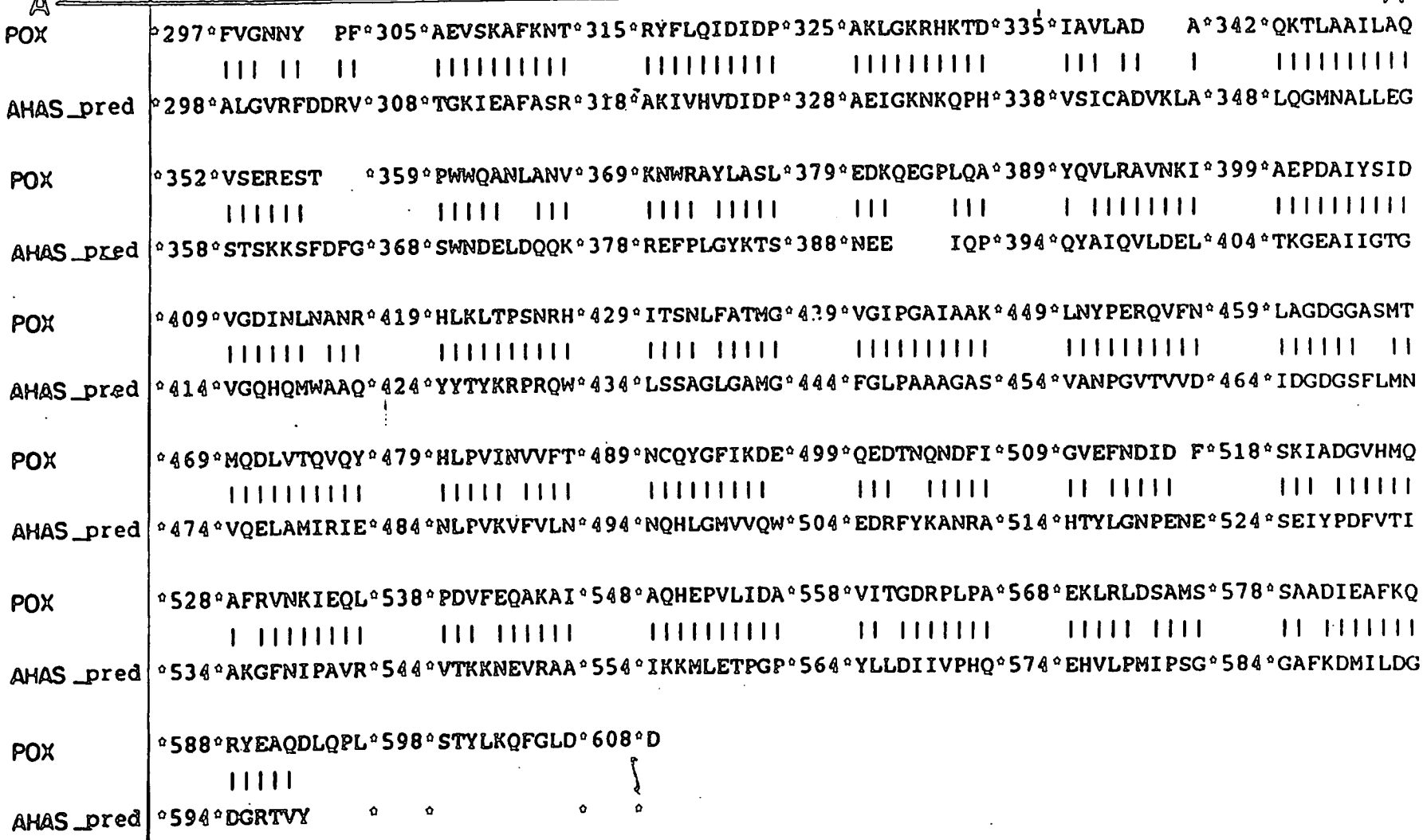

A
A
POX
AHAS_pred
POX
AHAS_pred
POX
AHAS_pred
POX
AHAS_pred
POX
AHAS_pred
POX
AHAS_pred

FIG. 3a

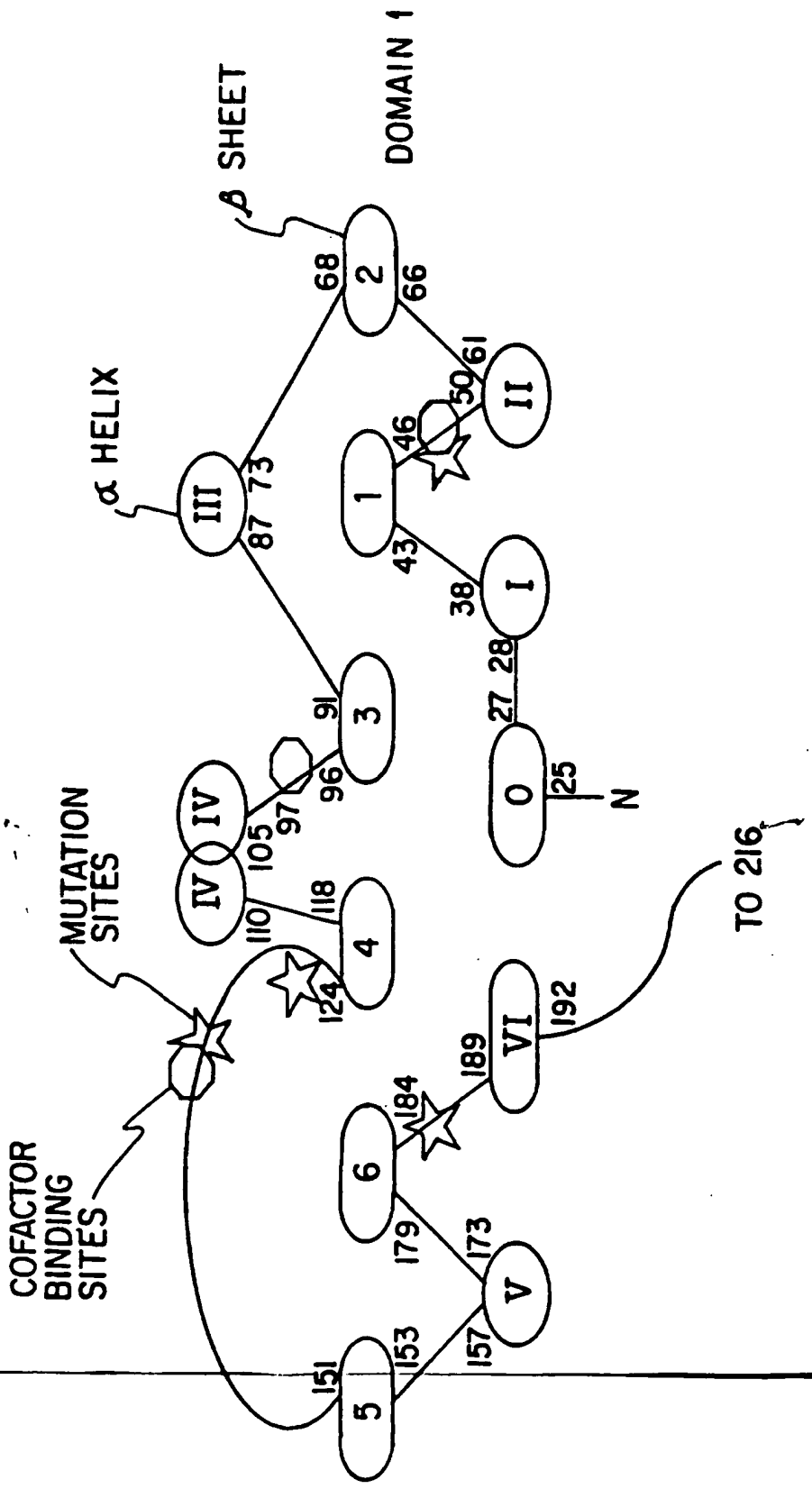

β SHEET
DOMAIN 1
α HELIX
MUTATION SITES
COFACTOR BINDING SITES
TO 216
N
I
II
III
IV
V
VI
0
1
2
3
4
5
6
68
66
61
50
46
73
87
43
38
91
96
97
105
110
118
124
27
28
25
189
184
192
179
173
153
157
151

FIG. 3b

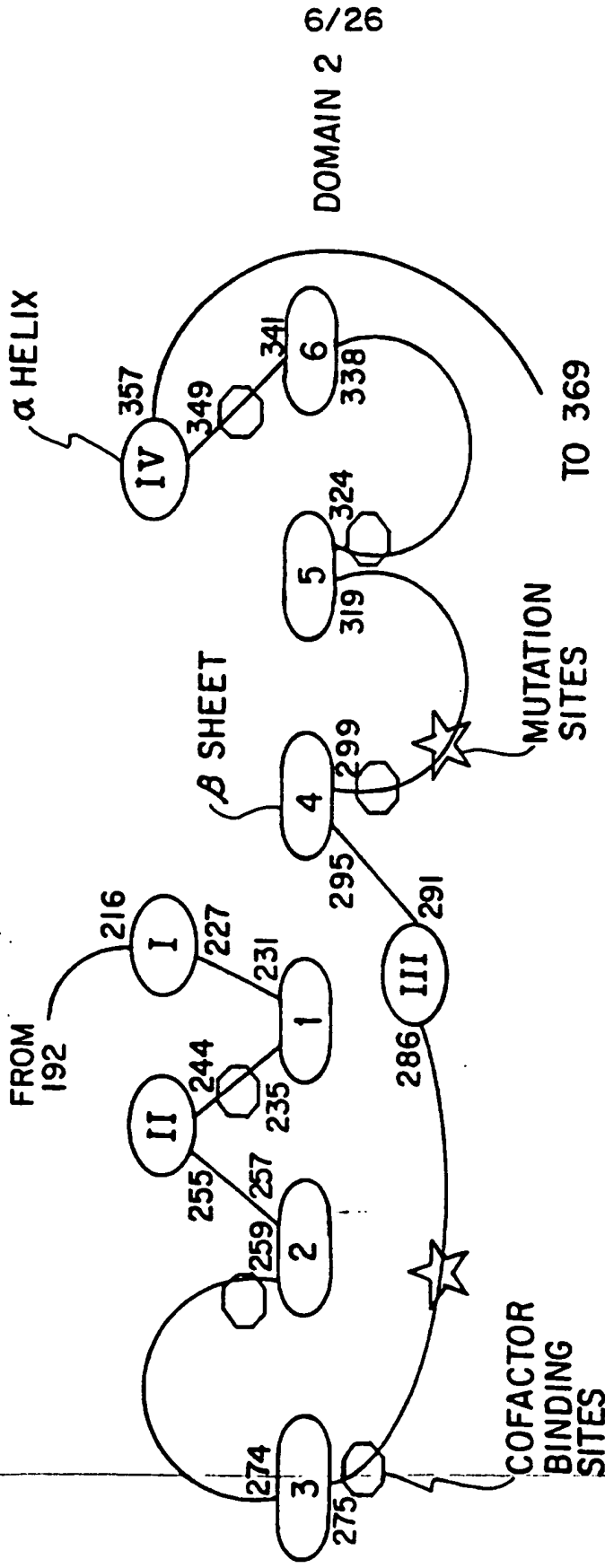

DOMAIN 2
α HELIX
β SHEET
FROM 192
TO 369
MUTATION SITES
COFACTOR BINDING SITES
I
II
III
IV
1
2
3
4
5
6
216
227
231
244
235
255
257
259
274
275
295
291
286
299
319
324
349
357
341
338

FIG. 3c

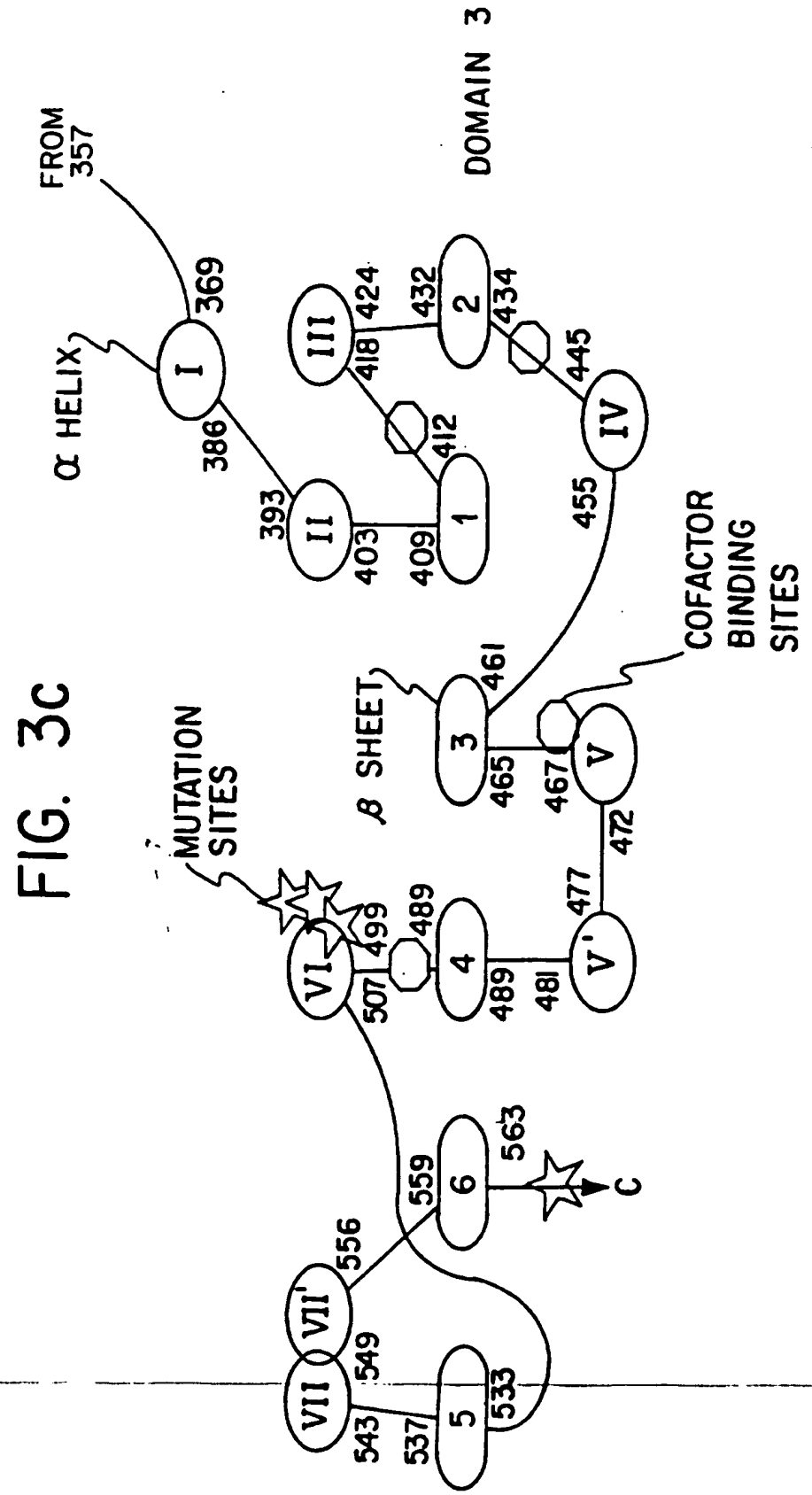

DOMAIN 3
FROM 357
α HELIX
β SHEET
MUTATION SITES
COFACTOR BINDING SITES
I
II
III
IV
V
V'
VI
VII
VII'
1
2
3
4
5
6
369
386
393
403
409
412
418
424
432
434
445
455
461
465
467
472
477
481
489
499
507
533
537
543
549
556
559
563
C

FIG. 4

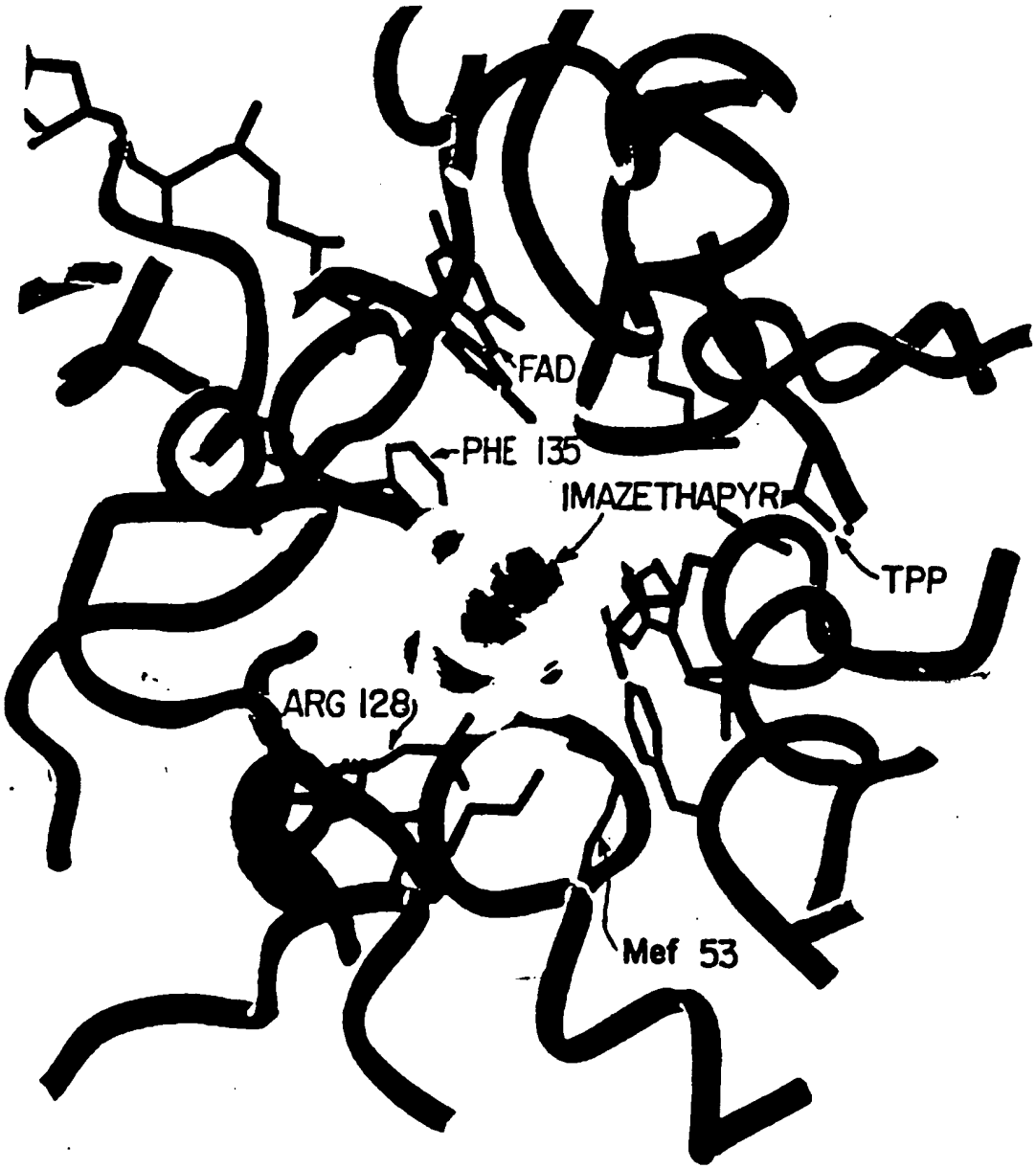


FIG. 5a

```
          1                                                  50
Pac751    .......... .......... .......... .......... ..........
Maizeals2 .......... .......... .......... ..MATAAAAS TALTGATTAA
Maizeals1 .......... .......... .......... ..MATAATAA AALTGATTAT
Tobac1    MAAA...APS PSSSAFSKTL SPSSSTSSTL LPRSTFPFPH HPHKTTPPPL
Tobac2    MAAA...AAA PSPS.FSKTL SSSSSKSSTL LPRSTFPFPH HPHKTTPPPL
Athcsr12  MAAATTTTTT SSSISFSTKP SPSSSKSPLP ISRFSLPFSL NPNKSSSSSR
Bnaal3    MAAA....TS SSPISLTAKP ...SSKSPLP ISRFSLPFSL TPQKPSSRLH
Bnaal2    .........M ASFSFFGTIP S.....SPTK ASVFSLPVSV TTLPSFPRRR
Consensus MAAA---ATS -S-SSFS--P SPSSSKSPT- -SRFTLPFS- TPLK--P---

          51                                                 100
Pac751    .......... .......... .GSAASPAMP MAPPATPLRP WGPTDPRKGA
Maizeals2 PKARRRAHLL ATRRALAAPI RCSAASPAMP MAPPATPLRP WGPTDPRKGA
Maizeals1 PKSRRRAHHL ATRRALAAPI RCSALSRATP TAPPATPLRP WGPNEPRKGS
Tobac1    HLTHTHIHIH SQRRRFTISN VISTNQKVSQ TEKTETFVSR FAPDEPRKGS
Tobac2    HLTPT..HIH SQRRRFTISN VISTTQKVSE TQKAETFVSR FAPDEPRKGS
Athcsr12  RRGIKSSSPS SISAVLNTTT NVTTTPSPTK PTKPETFISR FAPDQPRKGA
Bnaal3    R.......PL AISAVLNSPV NV....APEK TDKIKTFISR YAPDEPRKGA
Bnaal2    .........AT RVSVSANSKK DQDRTAS..R RENPSTFSSK YAPNVPRSGA
Consensus --TR-RAH-L -IRR-LN-PI --S-TS-A-P T-KP-TF-SR -APDEPRKGA
```

A——A

FIG. 5b

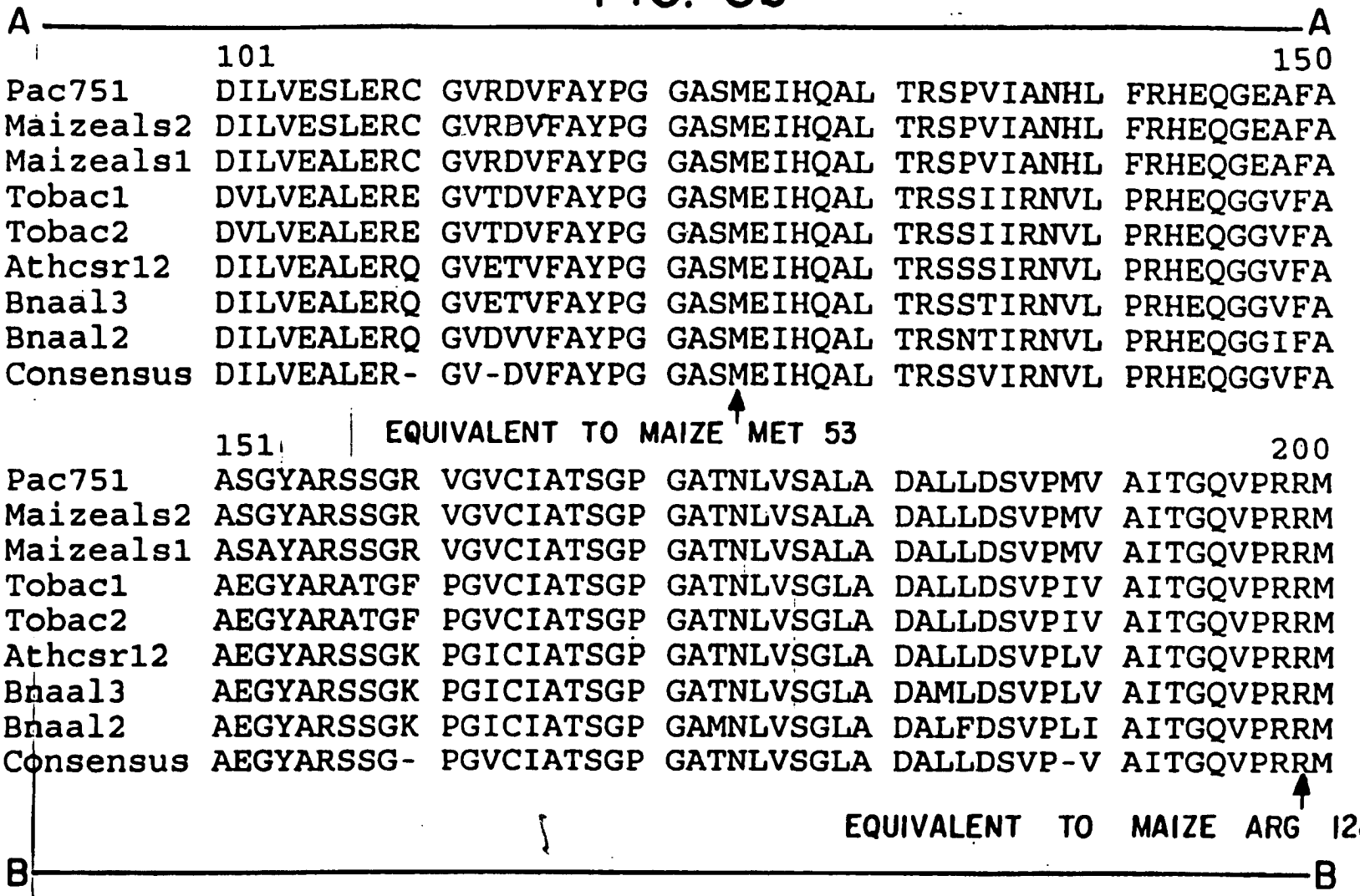

A
A
101
150
Pac751 DILVESLERC GVRDVFAYPG GASMEIHQAL TRSPVIANHL FRHEQGEAFA
Maizeals2 DILVESLERC GVRDVFAYPG GASMEIHQAL TRSPVIANHL FRHEQGEAFA
Maizeals1 DILVEALERC GVRDVFAYPG GASMEIHQAL TRSPVIANHL FRHEQGEAFA
Tobac1 DVLVEALERE GVTDVFAYPG GASMEIHQAL TRSSIIRNVL PRHEQGGVFA
Tobac2 DVLVEALERE GVTDVFAYPG GASMEIHQAL TRSSIIRNVL PRHEQGGVFA
Athcsr12 DILVEALERQ GVETVFAYPG GASMEIHQAL TRSSSIRNVL PRHEQGGVFA
Bnaal3 DILVEALERQ GVETVFAYPG GASMEIHQAL TRSSTIRNVL PRHEQGGVFA
Bnaal2 DILVEALERQ GVDVVFAYPG GASMEIHQAL TRSNTIRNVL PRHEQGGIFA
Consensus DILVEALER- GV-DVFAYPG GASMEIHQAL TRSSVIRNVL PRHEQGGVFA
EQUIVALENT TO MAIZE MET 53
151
200
Pac751 ASGYARSSGR VGVCIATSGP GATNLVSALA DALLDSVPMV AITGQVPRRM
Maizeals2 ASGYARSSGR VGVCIATSGP GATNLVSALA DALLDSVPMV AITGQVPRRM
Maizeals1 ASAYARSSGR VGVCIATSGP GATNLVSALA DALLDSVPMV AITGQVPRRM
Tobac1 AEGYARATGF PGVCIATSGP GATNLVSGLA DALLDSVPIV AITGQVPRRM
Tobac2 AEGYARATGF PGVCIATSGP GATNLVSGLA DALLDSVPIV AITGQVPRRM
Athcsr12 AEGYARSSGK PGICIATSGP GATNLVSGLA DALLDSVPLV AITGQVPRRM
Bnaal3 AEGYARSSGK PGICIATSGP GATNLVSGLA DAMLDSVPLV AITGQVPRRM
Bnaal2 AEGYARSSGK PGICIATSGP GAMNLVSGLA DALFDSVPLI AITGQVPRRM
Consensus AEGYARSSG- PGVCIATSGP GATNLVSGLA DALLDSVP-V AITGQVPRRM
EQUIVALENT TO MAIZE ARG 128
B
B

FIG. 5c

B — B

EQUIVALENT TO MAIZE PHE 135

	201				250
Pac751	IGTDAFQETP	IVEVTRSITK	HNYLVLDVDD	IPRVVQEAFF	LASSGRPGPV
Maizeals2	IGTDAFQETP	IVEVTRSITK	HNYLVLDVDD	IPRVVQEAFF	LASSGRPGPV
Maizeals1	IGTDAFQETP	IVEVTRSITK	HNYLVLDVDD	IPRVVQEAFF	LASSGRPGPV
Tobac1	IGTDAFQETP	IVEVTRSITK	HNYLVMDVED	IPRVVREAFF	LARSGRPGPI
Tobac2	IGTDAFQETP	IVEVTRSITK	HNYLVMDVED	IPRVVREAFF	LARSGRPGPV
Athcsr12	IGTDAFQETP	IVEVTRSITK	HNYLVMDVED	IPRIIEEAFF	LATSGRPGPV
Bnaal3	IGTDAFQETP	IVEVTRSITK	HNYLVMDVDD	IPRIVQEAFF	LATSGRPGPV
Bnaal2	IGTMAFQETP	VVEVTRTITK	HNYLVMEVDD	IPRIVREAFF	LATSVRPGPV
Consensus	IGTDAFQETP	IVEVTRSITK	HNYLVMDVDD	IPRVVQEAFF	LA-SGRPGPV

	251				300
Pac751	LVDIPKDIQQ	QMAVPVWDKP	MSLPGYIARL	PKPPATELLE	QVLRLVGESR
Maizeals2	LVDIPKDIQQ	QMAVPVWDKP	MSLPGYIARL	PKPPATELLE	QVLRLVGESR
Maizeals1	LVDIPKDIQQ	QMAVPAWDTP	MSLPGYIARL	PKPPATEFLE	QVLRLVGESR
Tobac1	LIDVPKDIQQ	QLVIPDWDQP	MRLPGYMSRL	PKLPNEMLLE	QIVRLISESK
Tobac2	LIDVPKDIQQ	QLVIPDWDQP	MRLPGYMSRL	PKLPNEMLLE	QIVRLISESK
Athcsr12	LVDVPKDIQQ	QLAIPNWEQA	MRLPGYMSRM	PKPPEDSHLE	QIVRLISESK
Bnaal3	LVDVPKDIQQ	QLAIPNWDQP	MRLPGYMSRL	PQPPEVSQLG	QIVRLISESK
Bnaal2	LIDVPKDVQQ	QFAIPNWEQP	MRLPLYMSTM	PKPPKVSHLE	QILRLVSESK
Consensus	LVDVPKDIQQ	QLAIPNWDQP	MRLPGYMSRL	PKPPA--LLE	QI-RL-SESK

C — C

FIG. 5d

C

```
          301                                                  350
Pac751    RPVLYVGGGC ARSGEELRRF VELTGIPVTT TLMGLGNFPS DD.PLSLRML
Maizeals2 RPVLYVGGGC AASGEELRRF VELTGIPVTT TLMGLGNFPS DD.PLSLRML
Maizeals1 RPVLYVGGGC AASGEELCRF VELTGIPVTT TLMGLGNFPS DD.PLSLRML
Tobac1    KPVLYVGGGC SQSSEDLRRF VELTGIPVAS TLMGLGAFPT GD.ELSLSML
Tobac2    KPVLYVGGGC SQSSEELRRF VELTGIPVAS TLMGLGAFPT GD.ELSLSML
Athcsr12  KPVLYVGGGC LNSSDELGRF VELTGIPVAS TLMGLGSYPC DD.ELSLHML
Bnaal3    RPVLYVGGGS LNSSEELGRF VELTGIPVAS TLMGLGSYPC ND.ELSLQML
Bnaal2    RPVLYVGGGC LNSSEELRRF VELTGIPVAS TFMGLGSYPC DDEEFSLQML
Consensus RPVLYVGGGC -NSSEELRRF VELTGIPVAS TLMGLG-FP- DD-ELSLRML

          351                                                  400
Pac751    GMHGTVYANY AVDKADLLLA LGVRFDDRVT GKIEAFASRA KIVHVDIDPA
Maizeals2 GMHGTVYANY AVDKADLLLA LGVRFDDRVT GKIEAFASRA KIVHVDIDPA
Maizeals1 GMHGTVYANY AVDKADLLLA FGVRFDDRVT GKIEAFAGRA KIVHIDIDPA
Tobac1    GMHGTVYANY AVDSSDLLLA FGVRFDDRVT GKLEAFASRA KIVHIDIDSA
Tobac2    GMHGTVYANY AVDSSDLLLA FGVRFDDRVT GKLEAFASRA KIVHIDIDSA
Athcsr12  GMHGTVYANY AVEHSDLLLA FGVRFDDRVT GKLEAFASRA KIVHIDIDSA
Bnaal3    GMHGTVYANY AVEHSDLLLA FGVRFDDRVT GKLEAFASRA KIVHIDIDSA
Bnaal2    GMHGTVYANY AVEYSDLLLA FGVRFDDRVT GKLEAFASRA KIVHIDIDST
Consensus GMHGTVYANY AVDKSDLLLA FGVRFDDRVT GKLEAFASRA KIVHIDIDSA
```

D

FIG. 5e

D

```
          401                                                  450
Pac751    EIGKNKQPHV SICADVKLAL QGMNALLEGS TSKKSFDFGS WNDELDQQKR
Maizeals2 EIGKNKQPHV SICADVKLAL QGMNALLEGS TSKKSFDFGS WNDELDQQKR
Maizeals1 EIGKNKQPHV SICADVKLAL QGMNTLLEGS TSKKSFDFGS WHDELDQQKR
Tobac1    EIGKNKQPHV SICADIKLAL QGLNSILESK EGKLKLDFSA WRQELTEQKV
Tobac2    EIGKNKQPHV SICADIKLAL QGLNSILESK EGKLKLDFSA WRQELTVQKV
Athcsr12  EIGKNKTPHV SVCGDVKLAL QGMNKVLENR AEELKLDFGV WRNELNVQKQ
Bnaal3    EIGKNKTPHV SVCGDVKLAL QGMNKVLENR AEELKLDFGV WRSELSEQKQ
Bnaal2    EIGKNKTPHV SVCCDVQLAL QGMNEVLENR RD..VLDFGE WRCELNEQRL
Consensus EIGKNKQPHV SICADVKLAL QGMN-VLE-- T-KLKLDFGS WRDELD-QKR

          451                                                  500
Pac751    EFPLGYKTSN EEIQPQYAIQ VLDELTKGEA IIGTGVGQHQ MWAAQYYTYK
Maizeals2 EFPLGYKTSN EEIQPQYAIQ VLDELTKGEA IIGTGVGQHQ MWAAQYYTYK
Maizeals1 EFPLGYKIFN EEIQPQYAIQ VLDELTKGEA IIATGVGQHQ MWAAQYYTYK
Tobac1    KHPLNFKTFG DAIPPQYAIQ VLDELTNGNA IISTGVGQHQ MWAAQYYKYR
Tobac2    KYPLNFKTFG DAIPPQYAIQ VLDELTNGSA IISTGVGQHQ MWAAQYYKYR
Athcsr12  KFPLSFKTFG EAIPPQYAIK VLDELTDGKA IISTGVGQHQ MWAAQFYNYK
Bnaal3    KFPLSFKTFG EAIPPQYAIQ VLDELTQGKA IISTGVGQHQ MWAAQFYKYR
Bnaal2    KFPLRYKTFG EEIPPQYAIQ LLDELTDGKA IITTGVGQHQ MWAAQFYRFK
Consensus KFPLG-KTFG E-IPPQYAIQ VLDELTKG-A IISTGVGQHQ MWAAQYY-YK
```

E

FIG. 5f

E — E

	501				550
Pac751	RPRQWLSSAG	LGAMGFGLPA	AAGASVANPG	VTVVDIDGDG	SFLMNVQELA
Maizeals2	RPRQWLSSAG	LGAMGFGLPA	AAGASVANPG	VTVVDIDGDG	SFLMNVQELA
Maizeals1	RPRQWLSSAG	LGAMGFGLPA	AAGAAVANPG	VTVVDIDGDG	SFLMNIQELA
Tobac1	KPRQWLTSGG	LGAMGFGLPA	AIGAAVGRPD	EVVVDIDGDG	SFIMNVQELA
Tobac2	KPRQWLTSGG	LGAMGFGLPA	AIGAAVGRPD	EVVVDIDGDG	SFIMNVQELA
Athcsr12	KPRQWLSSGG	LGAMGFGLPA	AIGASVANPD	AIVVDIDGDG	SFIMNVQELA
Bnaal3	KPRQWLSSSG	LGAMGFGLPA	AIGASVANPD	AIVVDIDGDG	SFIMNVQELA
Bnaal2	KPRQWLSSGG	LGAMGFGLPA	AMGAAIANPG	AVVVDIDGDG	SFIMNIQELA
Consensus	KPRQWLSSGG	LGAMGFGLPA	AIGA-VANP-	-VVVDIDGDG	SFIMNVQELA

	551				600
Pac751	MIRIENLPVK	VFVLNNQHLG	MVVQWEDRFY	KANRAHTYLG	NPENESEIYP
Maizeals2	MIRIENLPVK	VFVLNNQHLG	MVVQWEDRFY	KANRAHTYLG	NPENESEIYP
Maizeals1	MIRIENLPVK	VFVLNNQHLG	MVVQWEDRFY	KANRAHTFLG	NPENESEIYP
Tobac1	TIKVENLPVK	IMLLNNQHLG	MVVQWEDRFY	KANRAHTYLG	NPSNEAEIFP
Tobac2	TIKVENLPVK	IMLLNNQHLG	MVVQWEDRFY	KANRAHTYLG	NPSNEAEIFP
Athcsr12	TIRVENLPVK	VLLLNNQHLG	MVMQWEDRFY	KANRAHTFLG	DPAQEDEIFP
Bnaal3	TIRVENLPVK	ILLLNNQHLG	MVMQWEDRFY	KANRAHTYLG	DPARENEIFP
Bnaal2	TIRVENLPVK	VLLINNQHLG	MVLQWEDHFY	AANRADSFLG	DPANPEAVFP
Consensus	TIRVENLPVK	V-LLNNQHLG	MVVQWEDRFY	KANRAHTYLG	NP-NESEIFP

F — F

FIG. 5g

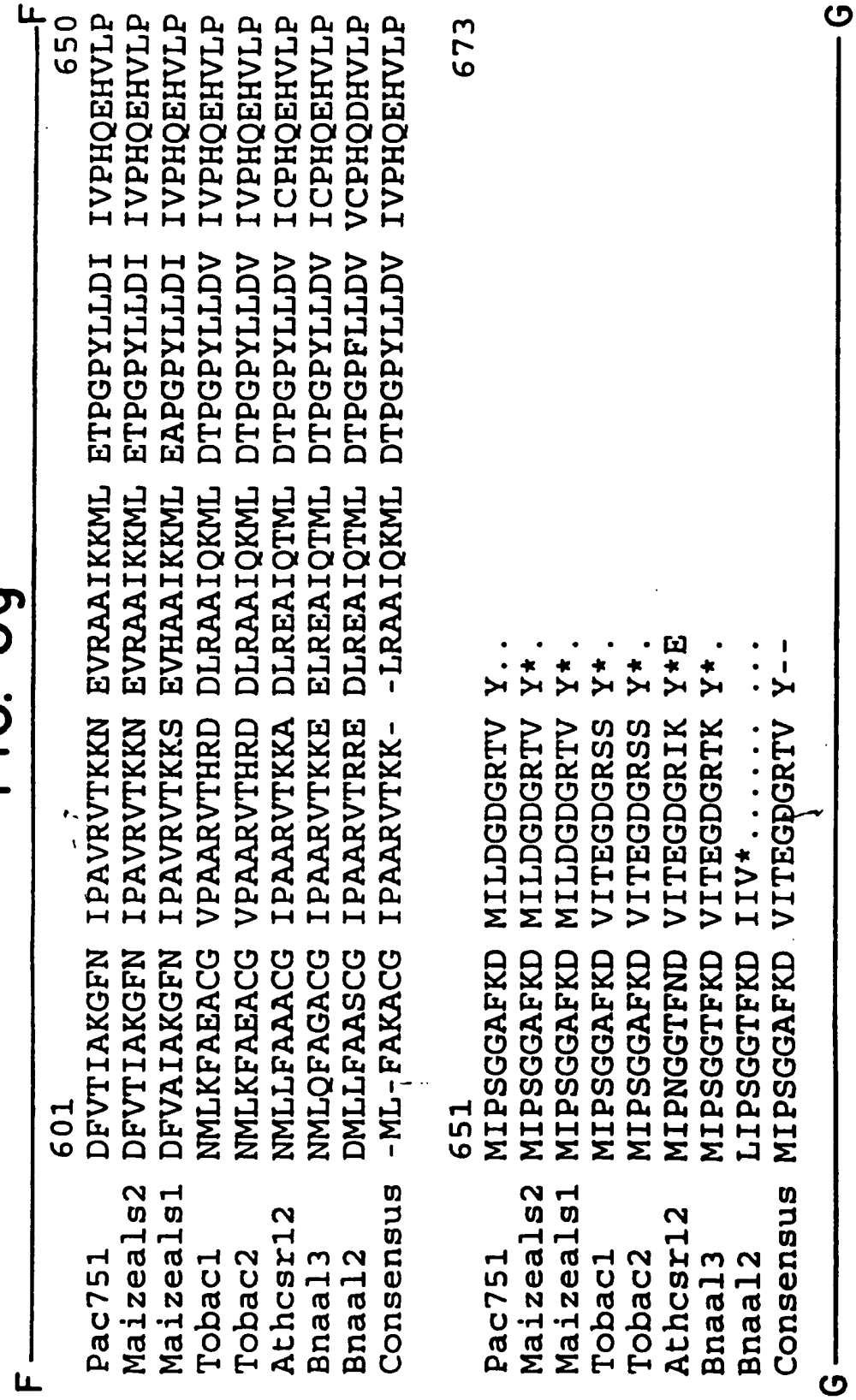

F
601
650
Pac751 DFVTIAKGFN IPAVRVTKKN EVRAAIKKML ETPGPYLLDI IVPHQEHVLP
Maizeals2 DFVTIAKGFN IPAVRVTKKN EVRAAIKKML ETPGPYLLDI IVPHQEHVLP
Maizeals1 DFVAIAKGFN IPAVRVTKKS EVHAAIKKML EAPGPYLLDI IVPHQEHVLP
Tobac1 NMLKFAEACG VPAARVTHRD DLRAAIQKML DTPGPYLLDV IVPHQEHVLP
Tobac2 NMLKFAEACG VPAARVTHRD DLRAAIQKML DTPGPYLLDV IVPHQEHVLP
Athcsr12 NMLLFAAACG IPAARVTKKA DLREAIQTML DTPGPYLLDV ICPHQEHVLP
Bnaal3 NMLQFAGACG IPAARVTKKE ELREAIQTML DTPGPYLLDV ICPHQEHVLP
Bnaal2 DMLLFAASCG IPAARVTRRE DLREAIQTML DTPGPFLLDV VCPHQDHVLP
Consensus -ML-FAKACG IPAARVTKK- -LRAAIQKML DTPGPYLLDV IVPHQEHVLP
651
673
Pac751 MIPSGGAFKD MILDGDGRTV Y..
Maizeals2 MIPSGGAFKD MILDGDGRTV Y*.
Maizeals1 MIPSGGAFKD MILDGDGRTV Y*.
Tobac1 MIPSGGAFKD VITEGDGRSS Y*.
Tobac2 MIPSGGAFKD VITEGDGRSS Y*.
Athcsr12 MIPNGGTFND VITEGDGRIK Y*E
Bnaal3 MIPSGGTFKD VITEGDGRTK Y*.
Bnaal2 LIPSGGTFKD IIV*..... ...
Consensus MIPSGGAFKD VITEGDGRTV Y--
G
G

FIG. 5h

G——————————————————————————————————————G

Pac751 - maize als2 AHAS isozyme as expressed from the pAC751 E. coli
expression vector (same as figure 1)
Maizeals2 - maize als2 AHAS isozyme (plant)
Maizeals1 - maize als1 AHAS isozyme (plant)
Tobac1 - tobacco AHAS SuRA isozyme (plant)
Tobac2 - tobacco AHAS SuRB isozyme (plant)
Athcsr12 - Arabidopsis thaliana Csr 1.2 AHAS gene (plant)
Bnaal3 - Brassica napus AHAS III isozyme (plant)
Bnaal2 - Brassica napus AHAS II isozyme (plant)

FIG. 6

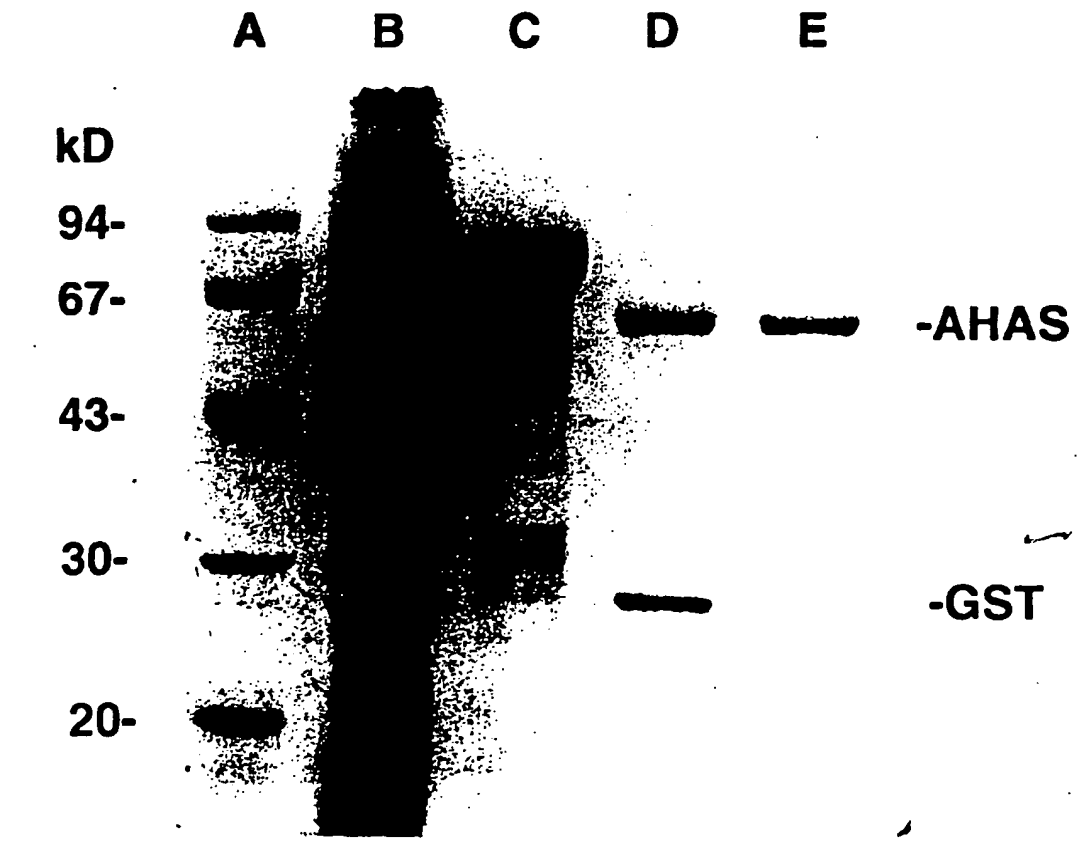


FIG. 7

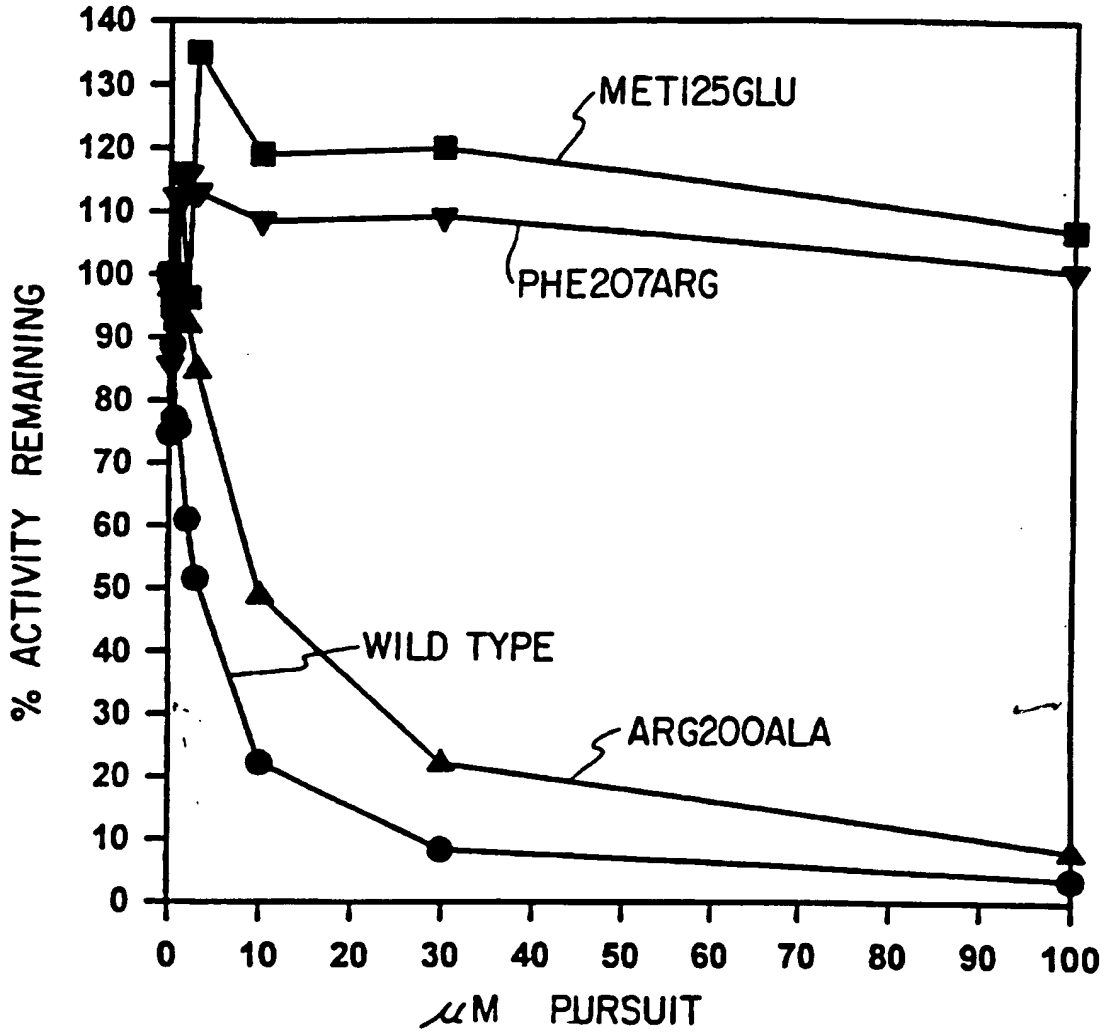

140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
% ACTIVITY REMAINING
MET125GLU
PHE207ARG
WILD TYPE
ARG200ALA
0
10
20
30
40
50
60
70
80
90
100
μM PURSUIT

FIG. 8

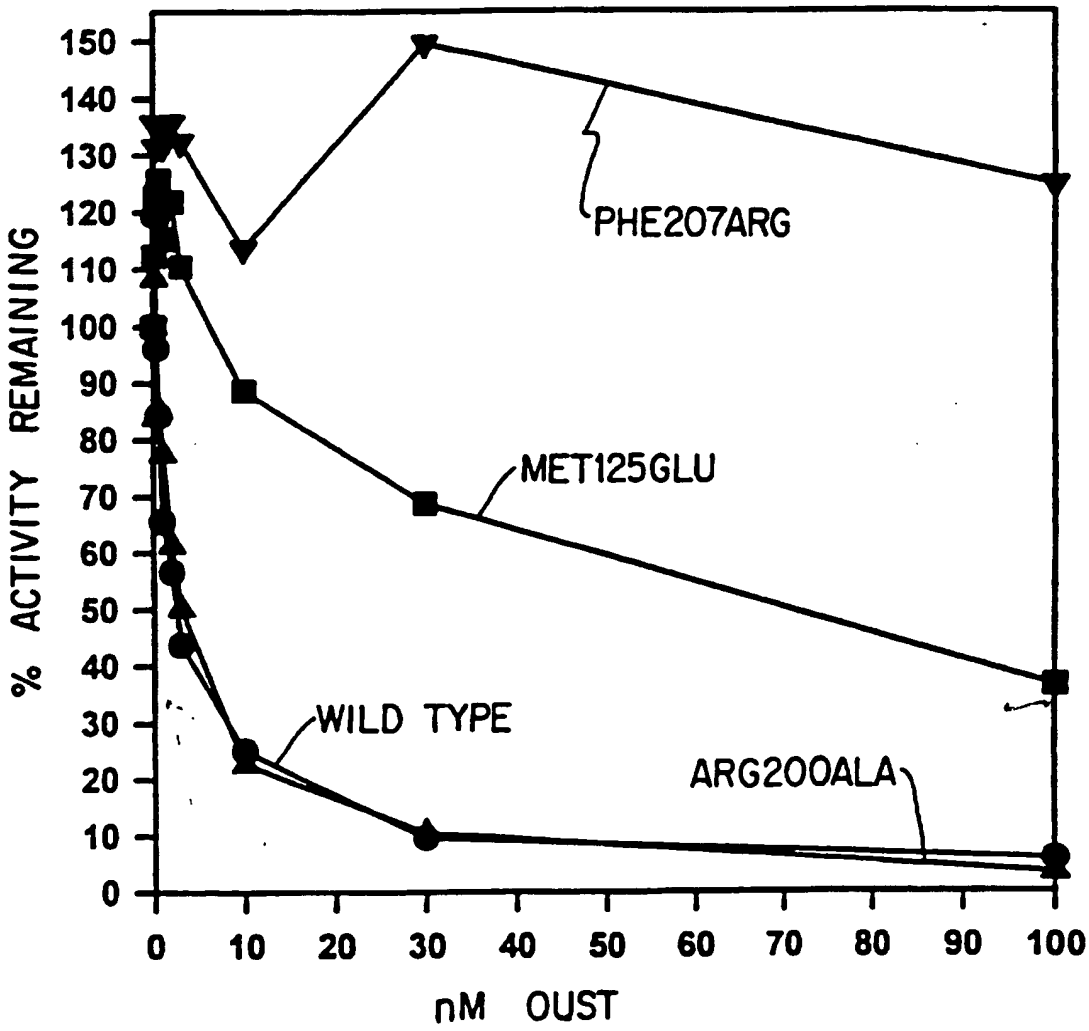

% ACTIVITY REMAINING
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
nM OUST
PHE207ARG
MET125GLU
WILD TYPE
ARG200ALA

FIG. 9

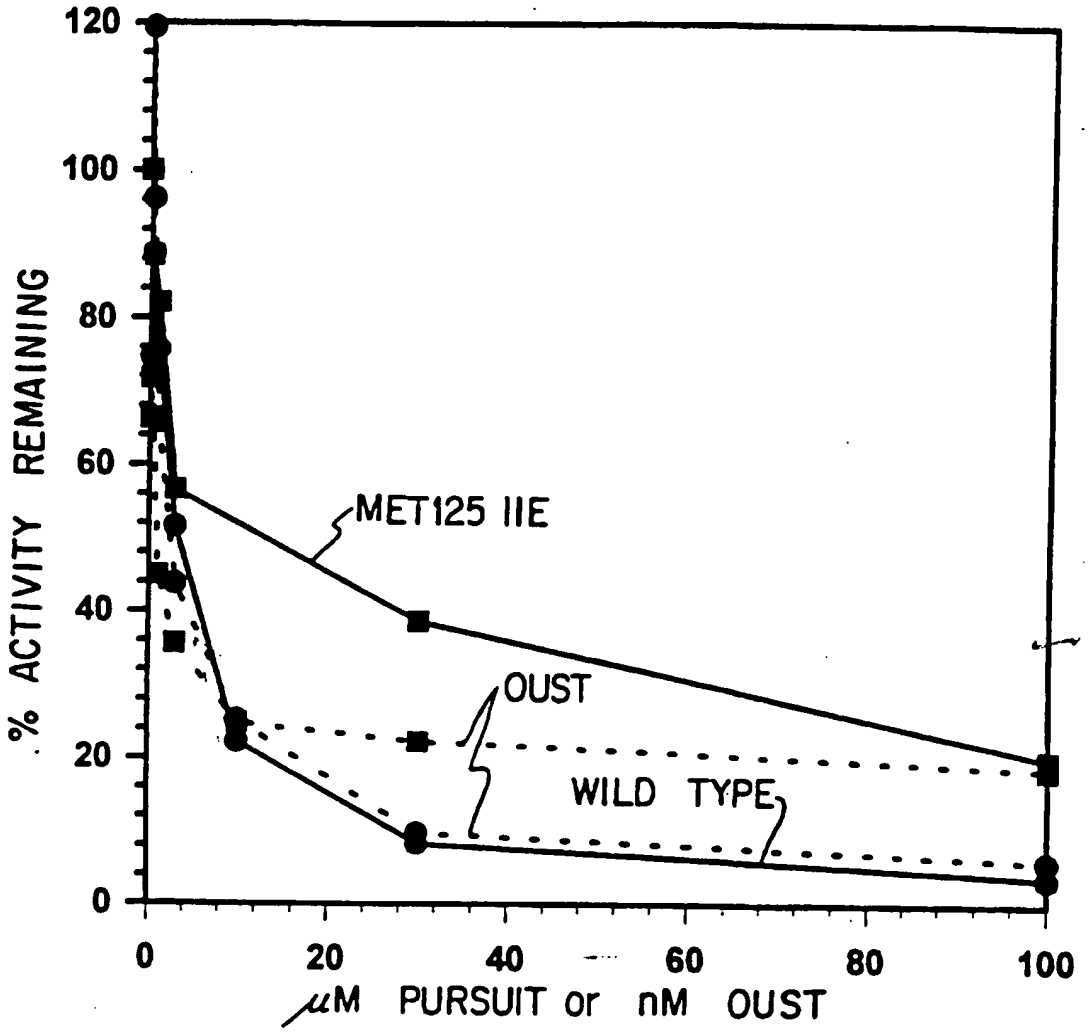

120
100
80
60
40
20
0
% ACTIVITY REMAINING
MET125 IIE
OUST
WILD TYPE
0
20
40
60
80
100
μM PURSUIT or nM OUST

FIG. 10

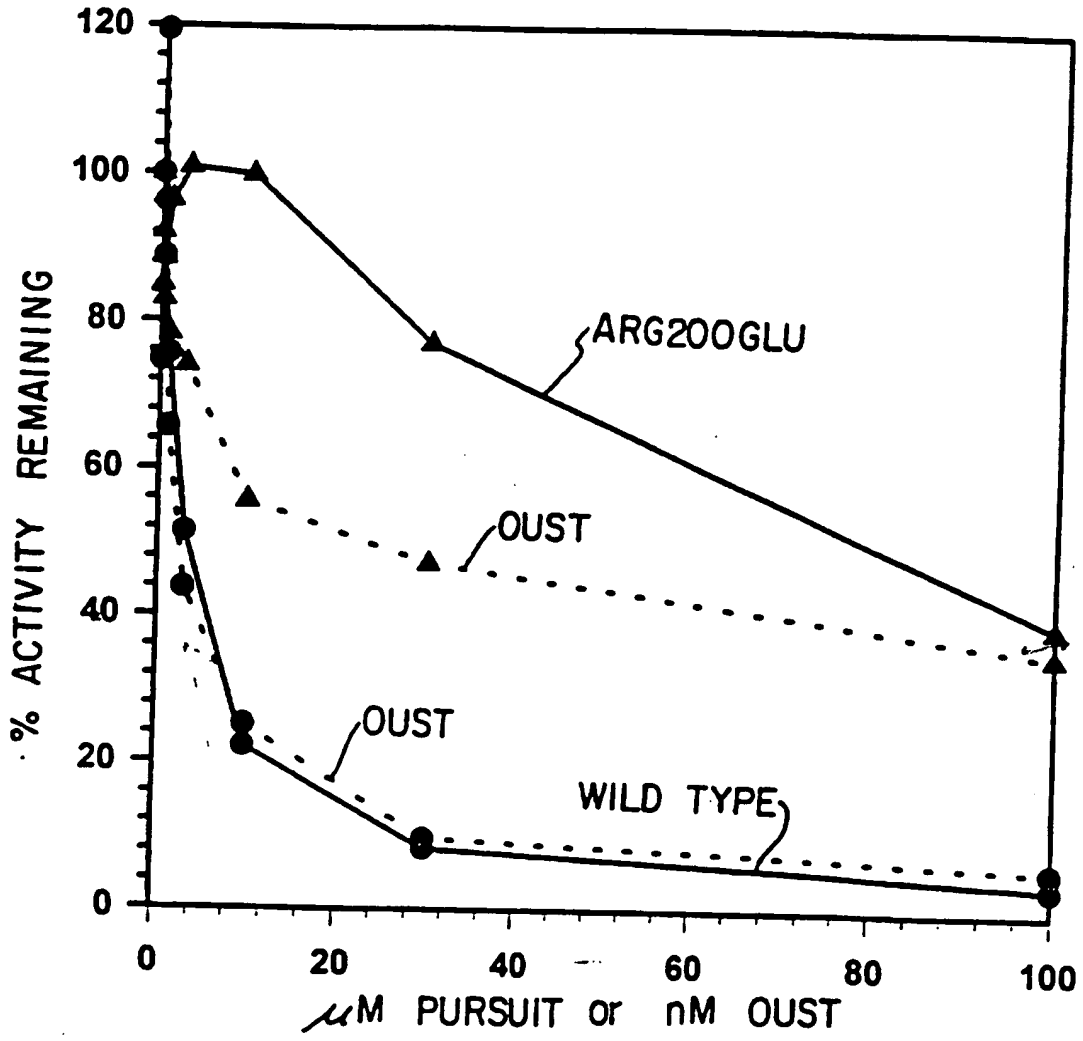

120
100
80
60
40
20
0
% ACTIVITY REMAINING
ARG200GLU
OUST
OUST
WILD TYPE
0
20
40
60
80
100
μM PURSUIT or nM OUST

FIG. 11

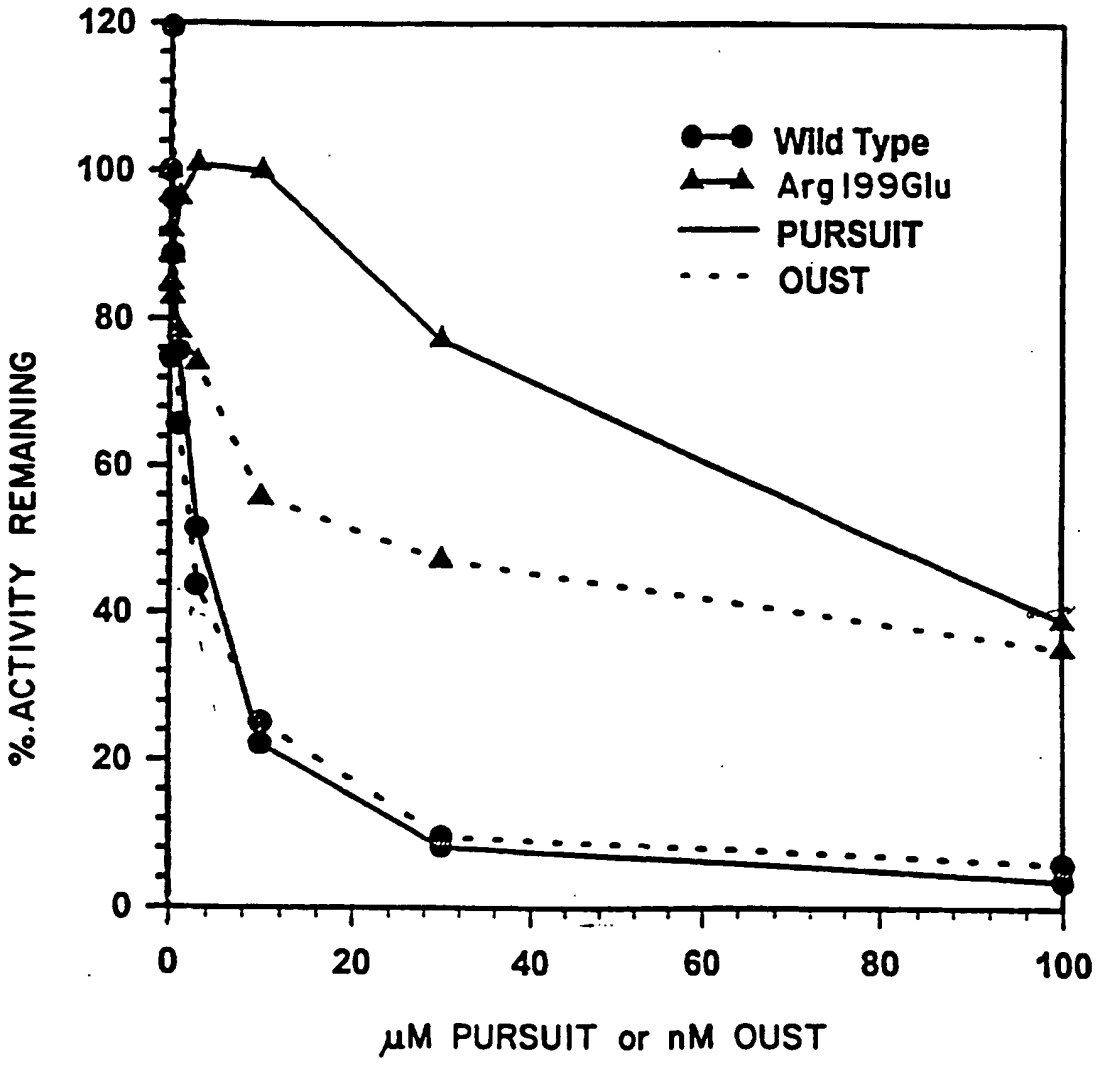

120
100
80
60
40
20
0
0
20
40
60
80
100
%.ACTIVITY REMAINING
µM PURSUIT or nM OUST
Wild Type
Arg199Glu
PURSUIT
OUST

FIG. 12

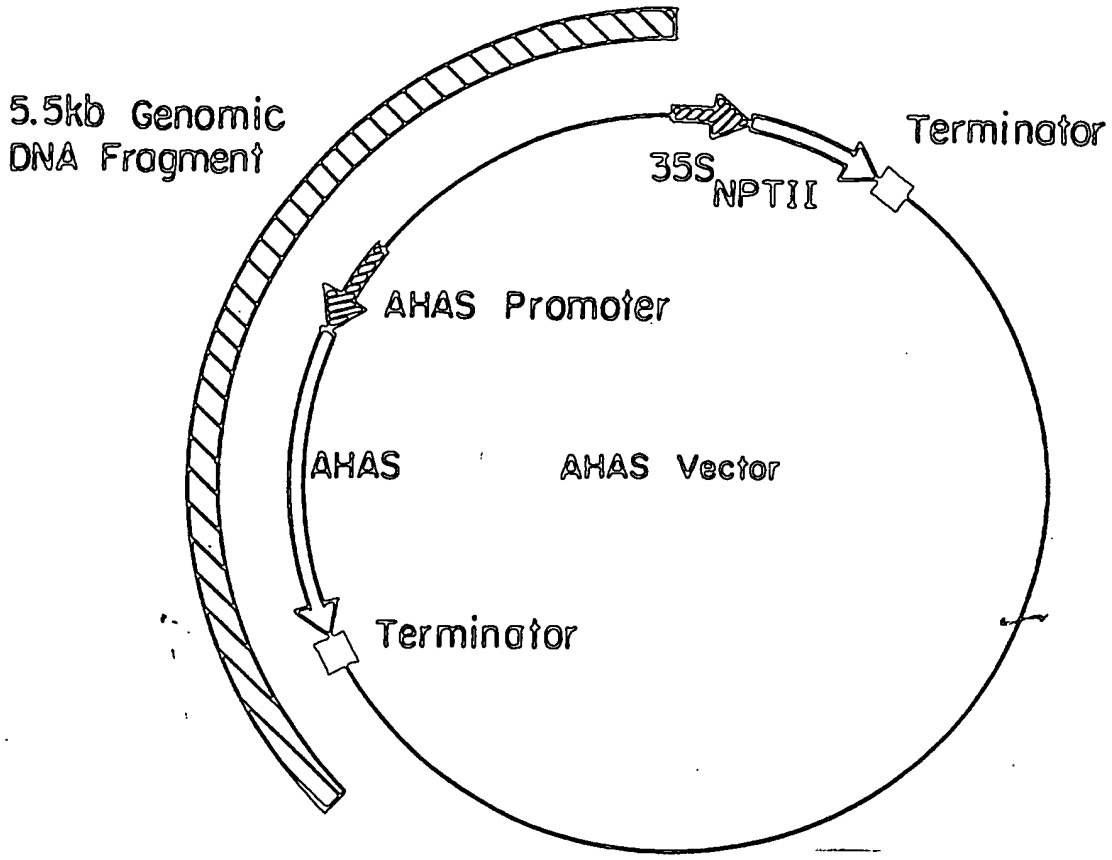

5.5kb Genomic
DNA Fragment
35S
NPTII
Terminator
AHAS Promoter
AHAS
AHAS Vector
Terminator

FIG. 13

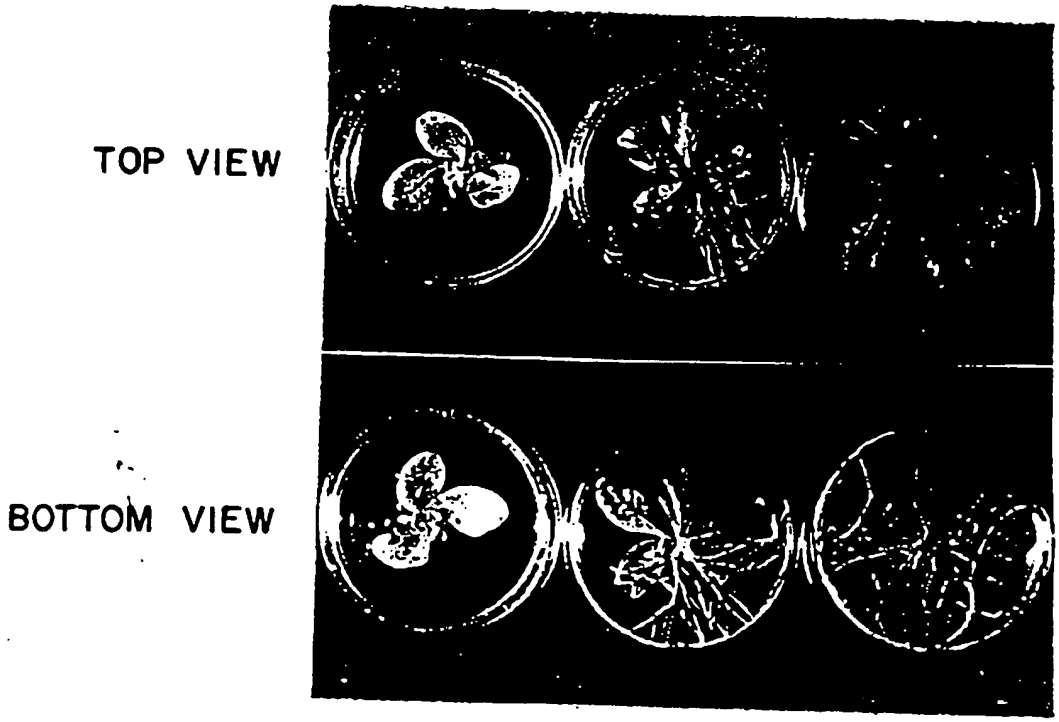


FIG. 14

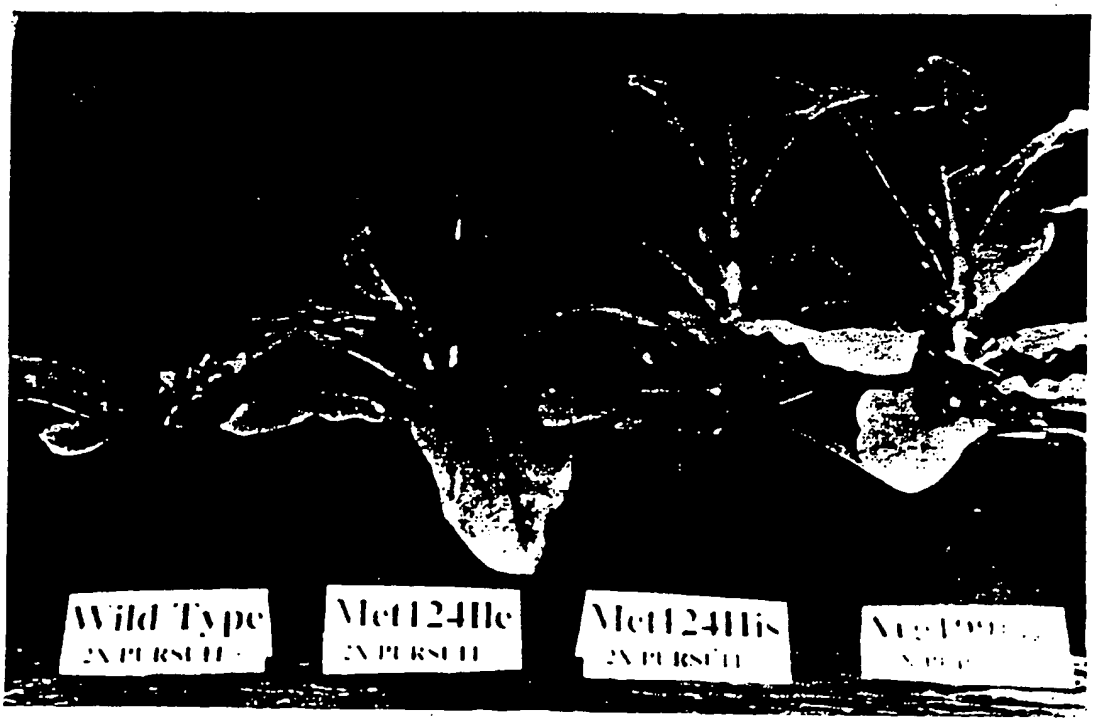
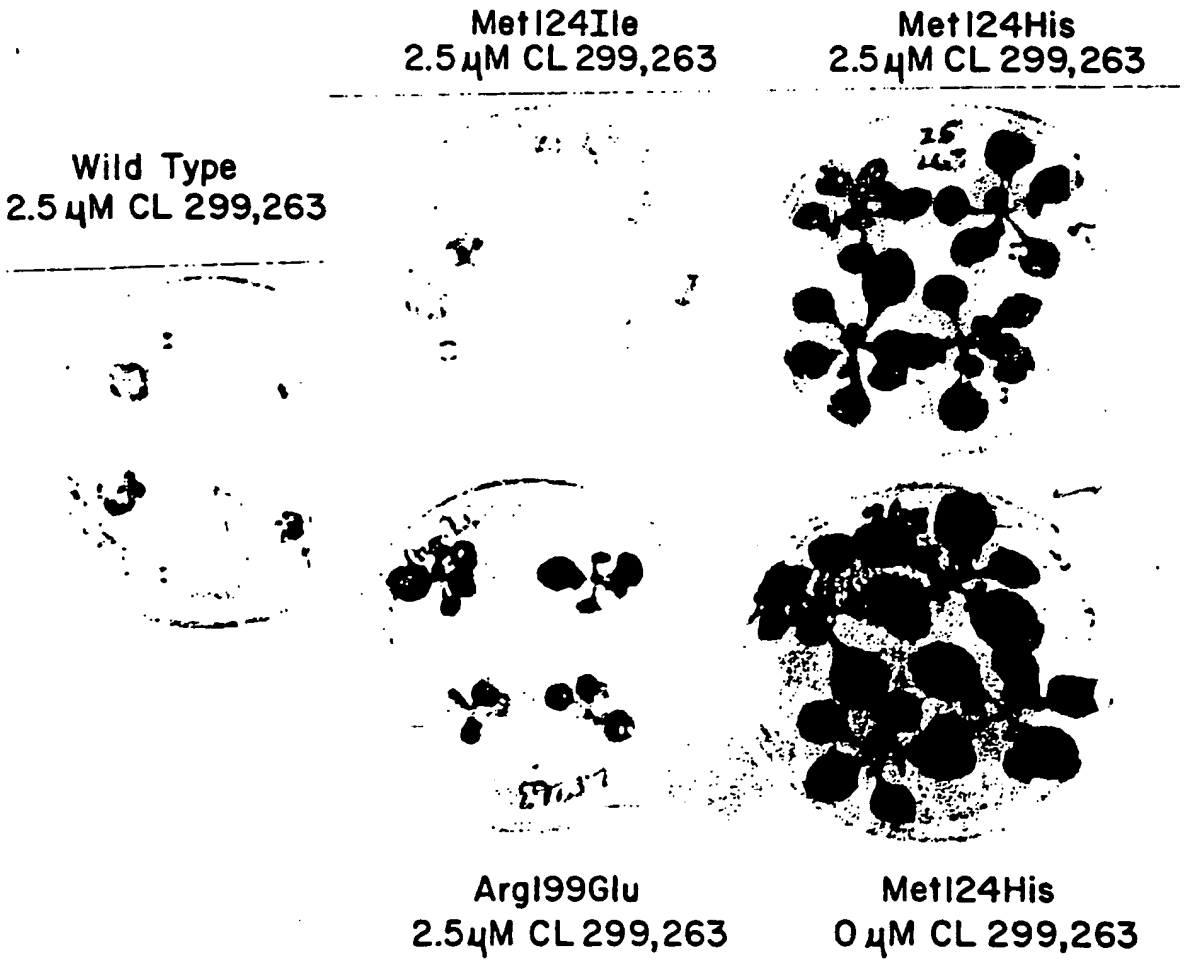


FIG. 15


Met124Ile
2.5 μM CL 299,263
Met124His
2.5 μM CL 299,263
Wild Type
2.5 μM CL 299,263
Arg199Glu
2.5μM CL 299,263
Met124His
0 μM CL 299,263

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05782

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 15/00, 9/88; A01H 5/00, 5/10
US CL :435/172.3, 232; 800/205
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/172.3, 232; 800/205

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, STN: medline, caplus, scisearch, lifesci, biosis, embase, wpids, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	MOSIMANN et al. A Critical Assessment of Comparative Molecular Modeling of Tertiary Structure of Proteins. Proteins: Structure, Function and Genetics November 1995, Vol. 23, pages 301-317. See from the beginning of the discussion section on page 311 to the end on page 316.	1-32
X --- A	BLUNDELL et al. Knowledge-based prediction of protein structures and the design of novel molecules. Nature 26 March 1987, Vol. 326, pages 347-352. See the entire article and in particular page 348, left column.	1-32 ------- 70-82

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

° Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 AUGUST 1996	18 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NASHAAT T. NASHED Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05782

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Protein Science, Vol. 3, Suppl. 1, July 1994, page 135, right column, the abstract number 479-S, IBDAH et al. MODEL FOR THE ACTIVE SITE OF ACETYLHYDROXY ACID SYNTHASE BASED ON HOMOLOGY MODELING AND DIRECTED MUTAGENESIS.	1-32 ------- 70-82
X, P ----- Y, P	BERNASCONI et al. A Naturaly Occurring Mutation Confers Broad Range Tolerance to Heribicde That Target Acetolactate Synthase. Journal of Biological Chemistry, 21 July 1995, Vol. 270, No. 29, pages 17381-178385. See abstract.	61, 62, 64 ---------- 1-57, 65-69
X --- Y	YADAV et al. Single amino acid substitutions in the enzyme acetolactate synthase confer resistance to the herbicide sulfometuron methyl. Proc. Natl. Acad. Sci. USA, June 1986, Vol. 83, pages 4418-4422. See particularly Figure 2 on page 4420.	61, 62, 64 ---------- 1-57, 65-69
X --- Y	SATHASIVA et al. Nucleotide sequence of a mutant acetolactate synthase gene from an imidazolinone-resitant Arabidopsis thaliana var. Columbia. Nucleic Acids Research 1990, Vol. 18, No. 8, page 2188. The entire article.	63 ---------- 18, 28, 36, 49, 57
X --- Y	US 5,013,659 A (BEDBROOK et al.) 07 May 1991. The entire document.	61, 62 ---------- 1-60, 63, 83, 84
Y	HAUGHN et al. Sulfonylurea-resistant mutants of Arabidopsis thaliana. Mol Gen Genet,1986, Vol. 204, pages 430-434. See abstract.	18, 28, 36, 49, 57, 63
X --- Y	HATTORI et al. Multiple resistance to sulfonylureas and imidazolinones conferred by an acetylhydroxyacid synthase gene with separate mutations for selective resistance. Mol Gen Genet. Vol. 232, 1992, pages 167-173. See entire article.	61-64 ---------- 1-60, 66-69, 83, 84
A	STIDHAM, Herbicides that Inhibit Acetohydroxyacid Synthase. Weed Science 1991, Vol. 39, 428-434.	70-82
Y	LAM et al. Rational Design of Potent, Bioavailable, Nonpeptide Cyclic Ureas as HIV Protease Inhibitors Science, 21 January 1994, Vol. 263, pages 380-384.	70-82
Y	WHITTLE et al. PROTEIN STRUCTURE-BASED DRUG DESIGN. Annu. Rev. Biophys. Biomol. Struct. 1994, Vol. 23, pages 349-375. The entire article.	70-82

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05782

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MULLER et al. Structure of Tiamine- and Flavin-Dependent Enzyme Pyruvate Oxidase. Science, 12 February 1993, Vol. 259, pages 965-967. The entire article.	1-32, 70-82

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05782

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/05782

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claims 1-43, 45-54, 56-67 and 83, drawn to a methods for the design of herbicide-resistant acetohydroxy acid synthase (AHAS), the mutants herbicide-resistant AHAS, their DNA sequences, vectors containing the DNA sequences and the cells containing the expression vectors.

Group II, claims 44, 55, 68, 69, and 84, drawn to herbicide-resistant plants and seeds and a method of controlling the growth of weeds.

Group III, claims 70-82, drawn to a method to design herbicides.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I comprises a method of protein/enzyme engineering to produce mutant enzymes that have full enzymatic activity, but have a reduced or no affinity to bind inhibitors of the wild-type enzyme. The special technical feature in the method of Group I is the protein design step, whereas that of Group II is the design of a small molecule that can bind best to the wild-type enzyme. Both special technical features of Groups I and II are different from that of Group III which is a herbicide resistant plants and seeds. Thus, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.1 so as to form a single invention concept.